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13. ABSTRACT (Maximum 200 Words)

We are investigating the role of inflammation in dopaminergic (DA) neuron death in the substantia nigra pars compacta (SNpc) of MPTP-treated mice. Following MPTP administration to mice, there is a robust microglial response in the SNpc that peaks earlier than the peak of SNpc DA neuronal death. Several enzymes are up-regulated or induced during the microglial response. Inducible nitric oxide synthase (iNOS), a principle enzyme in the synthesis of nitric oxide, is up-regulated in microglia in the SNpc following MPTP intoxication. Decreasing the presence of iNOS with the antibiotic minocycline, lessened the impact of MPTP on SNpc DA neurons. NADPH oxidase is also up-regulated in microglia. This enzyme is a major source of superoxide radical and is up-regulated significantly after MPTP administration. M40401, a manganese superoxide dismutase mimetic which can penetrate the blood brain barrier and which can react with the superoxide radical at rates equal to or greater than native SOD, significantly attenuated MPTP-induced SNpc DA neuronal death. We also saw less apoptotic cells in the SNpc of COX-2 knockout compared to their non-engineered littermates. Interestingly, thus far, this project has given credence to the oxidative stress theory of SNpc dopaminergic neuron death.

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Role of Inflammation in MPTP-Induced Dopaminergic Neuronal Death

2004 Report for Award Number DAMD-17-03-1-0002

(Period: December 1, 2003 to November 30, 2004)

Principle Investigator: Serge Przedborski, MD, PhD

Prepared by Vernice Jackson-Lewis, PhD

Introduction

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability), these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski, 2000). However, we do not know the etiology of this disorder. What we do know, aside from the death of the dopaminergic neurons in the SNpc, is 1) that PD affects mainly those individuals over 50 years of age (Fahn and Przedborski, 2000); 2) that there is a greater loss of dopaminergic terminals in the striatum than the loss of dopaminergic neurons in the SNpc (Fahn and Przedborski, 2000); 3) that there is an inflammatory component to PD that may be the cause of its progressive nature (McGeer et al, 1988; Banati et al, 1998); 4) that there is an up-regulation of certain cytokines in the SNpc (Mogi et al, 2000; Mogi et al, 1996; Mogi et al, 1994) and 5) that the superoxide radical and nitric oxide have been implicated in PD (DiMauro, 1993; Hunot et al, 1996). The central hypothesis that encompasses what we know is that following the initiation of the disease, a cascade of deleterious events leading to oxidative injury, macromolecule damage and evoked inflammation all conspire to produce mitochondrial dysfunction and energy failure which ends in the death of the dopaminergic neuron. In order to investigate the mechanisms that are involved in the death of the dopamine neuron in the SNpc, we used MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a neurotoxin that replicates, thus far, in humans (Langston et al, 1999), in non-human primates (Snow et al, 2000) and in other mammals such as mice (Jackson-Lewis et al, 1995), most of the hallmarks of PD. Like in PD, MPTP causes a greater loss of dopaminergic terminals in the striatum than of dopaminergic neurons in the SNpc (Moratalla et al, 1992). Also noted, is that MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area of the brain (Muthane et al, 1994). Furthermore, as in PD, MPTP causes a selective defect in mitochondrial electron transport chain trafficking such that there is a blockage at the complex 1 site of the mitochondrial electron transport chain (Gluck et al, 1994) which suggests an overproduction of free radicals.

Aside from the severe loss of dopaminergic neurons in the SNpc, PD exhibits a marked gliosis in this area of the brain. Astrocytes and microglia, the two main components of the glial response are both up-regulated (Langston et al, 1999), however, the magnitude of their responses is quite different. The astrocytic response, both in number and in immunoreactivity, is, in general, mild and in only a few instances, has this response been dramatic (Forno et al, 1992; Mirza et al, 2000). In contrast, the activation of microglia has been consistently strongest in the SNpc the area of the brain most affected by the neurodegenerative process (Vila et al, 2001). Moreover, activated microglia are found in close proximity to the remaining dopaminergic neurons (McGeer et al, 1988), around which they sometimes cluster to produce what resembles neurophagia. This same situation has been noted in the three autopsied brains that were recovered from those individuals who self-administered MPTP (Langston et al, 1999). Microglial activation and neurophagia are indicative of an active ongoing process of cell death which is consistent with the progressive nature of PD. Yet, data from PD and from the few individuals who injested MPTP do not provide us with information about the temporal

relationship between SNpc dopaminergic neuron death and microglial activation. To this end, we have used the MPTP mouse model of PD to glean information about and to sort out the process of gliosis as it relates to the time course of microglial and astrocytic activation in SNpc dopaminergic neuron death. We have found the while microglia are up-regulated early on in the MPTP neurotoxic process and reaches maximum before the peak of dopamine neuron death in the SNpc, astrocytes, mildly activated early on, are further up-regulated and remain up-regulated for an lengthy period of time following MPTP administration (Liberatore et al, 1999). Thus, it is reasonable to think that glia participate in the death of the dopaminergic neurons in the SNpc both in PD and in the MPTP mouse model of PD.

Microglial cells are resident macrophage cells in the brain that have the ability to react promptly in response to brain injury and to subtle changes in the microenvironment surrounding their charges, the neurons (Kreutzberg, 1996). In the normal brain, microglial cells are in a resting state in which they are barely visible and very few, if any, ramified processes are detected. In contrast, in a pathological situation, microglia quickly proliferate, become hypertrophic, increase in size to resemble "spiders" and produce a number of marker molecules that are either pro- or anti-inflammatory in nature. Thus, microglial cells are a double-edged sword type of cell, producing such proinflammatory compounds as the superoxide radical, nitric oxide (NO), prostaglandin E_2 (PGE_2), excitatory amino acids, and proinflammaroty cytokines such as interleukin-1-beta (IL-1-ß), or such anti-inflammatory compounds as interleukin-4 (IL-4) and transforming growth factor-beta-1 (TGF- ß1) (Banati et al, 1993; Gehmann et al, 1995; Hopkins and Rothwell, 1995). Since activated microglia have been found in PD and in the MPTP mouse model of PD, and microglia can produce such damaging molecules as mentioned above, attempts to block their activation seem reasonable. Using minocycline, a second generation tetracycline antibiotic, we demonstrated that this antibiotic can block microglial activation which in turn, decreases SNpc dopamine neuron death (Wu et al, 2002). This effect is apparently independent of its antibiotic activity. Thus, it is important to elucidate the mechanism of microglial activation in PD and in the MPTP mouse model of PD as well as continue our efforts to identify compounds that exhibit neuroprotective qualities in the SNpc. Since this is a Program Project with several components, we will concern ourselves with the Project Core (Core A and Core B) and Project .1 (Przedborski).

Body of the Research.

Our long-term goal is the study of the pathogenesis of PD based on the oxidative stress hypothesis of PD. Our recent findings of microglial activation during the most active phase of neuronal degeneration in the MPTP mouse model of PD has led us to believe that microglial activation is an integral part of the neurodegenerative process in PD. and in the MPTP mouse model of PD. To this end, elucidating the mechanisms of microglial activation will help us identify possible therapies which may improve the symptomology of or to stop the progression of PD. Along these lines, using the oxidative stress theory of PD, our research plan may help us to define a primary role for inflammation in the neurotoxic process following MPTP administration which, in all likelihood, may be the same situation for PD. In **Specific Aim I**, we propose to determine the role of microglial

activation in the MPTP neurotoxic process. Our plan is to administer different doses of MPTP to mice pretreated with different doses of minocycline, a drug known to block microglial activation then assess neuroprotection in the SNpc using HPLC, immunstaining and Western blot analyses. We also plan to use primary mixed neuronal/microglial cultures to study more definitively the role of activated microglia in MPTP-mediated DA neuronal death and the contribution of proinflammatory factors. We will also assess, by pharmacological intervention, the beneficial effects of inhibiting such proinflammatory factors. In **Specific Aim II**, we propose to define the role of NADPH oxidase in the MPTP neurotoxic process at different time points in MPTP-treated mice that are deficient in NADPH oxidase We will assess neuroprotection and microglial activation as in Specific Aim I. Since both microglial NADPH oxidase and dopamine neurons stimulate superoxide radical during MPTP toxicity, in **Specific Aim III**, we propose to assess the dose and time-dependent neuroprotective effect of M40401, a superoxide disputase mimetic, in the SNpc of MPTP-treated mice. Another component of the inflammatory response in PD is the up-regulation of the COX-2 enzyme which is one of the enzymes in the synthesis of prostaglandin E_2 (PGE_2), thus, **Specific Aim IV** proposes to examine the contribution of prostaglandin PGE_2 to the MPTP neurotoxic process by assessing the roles of the PGE_2-synthesizing enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2 in the MPTP neurotoxic process in different brain regions and at different time points using different doses of the toxin. We plan to use COX-1 and COX-2 knockout mice for these studies. The planned specific aims should provide valuable information about the mechanisms involved in the inflammatory response related to the MPTP neurotoxic process and to PD as well as identify targets for therapeutic intervention.

Key Research Accomplishments

Core A.

Core A is the administrative arm of the Parkinson's Disease Research Program. It provides the centralized scientific leadership necessary for this program to succeed. The members of Core A continue to review findings and discuss them in light of the proposed program of research.

Core B.

Core B is the centralized MPTP facility which is located on the 19th floor of the College of Physicians and Surgeons here at Columbia University. Its role is to support the research activities of all three projects. Four specific aims constitute the work of Core B all based on supplying the needed MPTP-treated mice for this program project Mice are received and housed by animal care technicians, then placed outside of the MPTP Facility. Core B is informed of their arrival and a member of the Core B staff puts the housed mice into the MPTP room. The delivery is logged in and the animals are left to acclimate for one week prior to any injection schedule. Experiments for the tissues necessary for the Program Project are discussed, worked out and scheduled with Dr. V. Jackson-Lewis. Injections are performed by Dr. Jackson-Lewis. Samples are then collected according to the scheduled experiment, and are either used here at Columbia or sent as per the scheduled experiments to the individual who needs them. No glitches in shipping samples have occurred thus far. To insure that samples are prepared properly, quality controls are run frequently with

experimental samples on the HPLC. Research fellows here at Columbia are trained on how to handle MPTP samples by Dr. Jackson-Lewis. All research fellows who handle the MPTP-treated mice are also required to read our paper regarding the safe handling of MPTP mice and samples (ref) and to be trained in the handling of MPTP samples by Jackson-Lewis. This year, a number of individuals from institutions such as Woods Hole, have visited our facility to see how an MPTP facility should operate. Also, inspection by AALAC and the university's Institutional Animal Care and Use Committee found no deficiencies in this facility. In fact, has used our facility several times to demonstrate how an independent facility should operate.

Specific Aim I.

In Vivo Experiments.

The work using minocycline as a neuroprotective agent in the MPTP mouse model of PD is complete. We have noted that during the MPTP-induced inflammatory response in the SNpc of mice, microglia exhibit immunostaining which indicates the presence of the inducible form of nitric oxide synthase (iNOS). In these experiments, we have demonstrated that minocycline, a second generation tetracycline antibiotic, reduces the up-regulation of inducible nitric oxide synthase and increases the number of surviving dopaminergic neurons in the SNpc. Our results have been published in the Journal of Neuroscience under the title **Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson disease. See attached manuscript 1.** In future studies, we plan to use minocyline in combination with a SOD mimetic (M40401) to ascertain the effects of blocking both the overproduction of the superoxide radical and the induction of NO in the MPTP mouse model of PD.

Cell Culture Experiments for Specific Aim I.

We have moved into our renovated laboratories with its own cell culture facility and are now in the process of establishing the microglial/neuronal cultures necessary for the proposed project and the conditions in which to keep them viable. These microglial/neuronal cultures will be used to sort out the contribution of activated microglia to the MPTP neurotoxic process.

Specific Aim II.

Recent studies using post mortem samples from the brains of individuals who had injected street preparations of a synthetic heroin contaminated with MPTP demonstrated microglial activation which suggests that inflammation may be a part of the neurodegenerative process (ref). Consistent with this is the fact that experimental models of PD including the MPTP model show a significant microglial activation (Czlonkowska et al, 1996; Liberatore et al, 1999) which suggests that inflammatory factors either initiate or modulate SNpc DA neuronal death (Gao et al, 2002; Wu et al, 2002). Among the inflammatory mediators capable of promoting DA neurodegeneration are microglial-derived reactive oxygen species (ROS) which probably contribute to the oxidative stress that is reportedly part of the oxidative stress hypothesis of PD (Babior, 1999). A significant source of ROS during inflammation is NADPH oxidase. This multimeric enzyme is composed of 4 subunits

(GP^{91phox}, p_{22phox}, p^{47phox} and p^{40phox}) and is inactive in resting microglia because p^{47phox}, p^{67phox} and p^{40phox} are all present in the cytosol as a complex and are separated from the transmembrane proteins, GP^{91phox} and p^{22phox} (Babior, 1999). When microglia become activated, p^{47phox} is phosphorylated and the entire complex translocates to the plasma membrane where it assembles with GP^{91phox} and p^{22phox} to form the NADPH oxidase complex. which is now capable of reducing oxygen to the superoxide radical (Babior, 1999). The superoxide radical, in turn, gives rise to secondary reactive oxygen species (Babior, 1999) We have completed Specific Aim II of the proposed studies and have published our results in a manuscript entitled **NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease** which has been published in the Proceedings of the National Academy of Science. **See attached manuscript 2.** At this point, we are trying to further characterize the GP^{91} knockout mice.

Specific Aim III

In Specific Aim III of Project 1, we evaluated the contribution of the superoxide radical to the demise of dopaminergic neurons in the SNpc of mice after MPTP challenges. Superoxide, the product of the one electron reduction of oxygen by NADPH oxidase and other flavoproteins is thought to play a central role in the injury of neurons after MPTP administration. It is now well appreciated that, under normal circumstances, the biological reactivity of the superoxide radical is kept under the control of superoxide dismutase (SOD) enzymes. These enzymes include the mitochondrial located manganese (Mn) enzyme and the copper/zinc (Cu/Zn) enzyme present in the cytosol or extracellular spaces. Uncontrolled overproduction of superoxide, as it suggested to occur after MPTP administration and subsequent inflammation, that overwhelms the antioxidant defenses could create a significant oxidative burden. Super oxide, by oxidizing iron-sulfur clusters or after conversion to hydrogen peroxide and peroxynitrite, could significantly injure macromolecules and eventually lead to neuron death. The central tenant of this third specific aim was to evaluate the employment of a superoxide dismutase mimetic (SODm), M40401, and its inactive SODm congeners M40404 and M404035. M40401 is a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native superoxide dismutase enzymes but with the advantage of being a much smaller molecule (MW 483 versus MW 30,000 for the mimetic and native enzyme, respectively) and being able to penetrate the blood brain barrier. Furthermore, M40401 is stable in vivo, penetrates cells readily, has wide tissue distribution in rats and mice, and is excreted intact with no detectable dissociation in urine and feces.

The typical MPTP administration, mice (n= 6-8 per group and per condition) receiveing four intraperitoneal (i.p.) injections of MPTP-HCL, 20 mg/kg free base, in saline at 2 hour intervals was used. For M40401, M40404 and M40435 treatments, mice received a single subcutaneous (s.c.) injection of either compound dissolved in water (pH adjusted to7.5 with 0.2M $NaHCO_3$ in varying doses (1.875 to 15mg/kg) 30 min prior to the first MPTP injection, then once daily for four additional days. Control mice received i.p. saline/s.c. water (pH 7.5) only. Mice (n =4-7 per group; saline-water, M40401-saline, water-MPTP and M40401-MPTP) were sacrificed at selected time points and their brains were used for morphological and biochemical studies. All procedures were in accordance with the

National Institute of Health guidelines for the use of live animals and were approved by the Institutional Animal Care and Use Committee of Columbia University. MPTP handling and safety measures were in accordance with our published procedures.

SUMMARY OF RESULTS:

Administration of M40401 attenuated dopamine neuron death in the substantia nigra pars compacta (SNpc) of MPTP-treated mice. The two M40401 inactive analogs, M40404 and M40435, at doses equal to those of M40401, were not effective in protecting SNpc dopaminergic neurons from the toxic insult by MPTP. Treatment with M40401 significantly reduced the levels of superoxide dependent oxidation of hydroethidine, as well as the levels of proteins modified by reactive carbonyls and tyrosine nitration in the striatum of MPTP-treated mice. Protein carbonylation and tyrosine nitration are well-recognized biomarkers of oxidative modification of proteins. M40401 also reduces the microglial response to MPTP in the SNpc of mice. This finding is consistent with the previously reported pro-inflammatory role of superoxide. The preliminary data gathered thus far provide support for the central role of superoxide in the injury associated with the administration of MPTP. Removal of superoxide by the use of a cell preamble mimetic protected from neuron death and attenuated the increases in protein oxidative medications and inflammation. These provide sound and direct evidence for the suspected role of superoxide-driven oxidative pathways in parkisonian neurodegeneration. **A manuscript of the work performed in Specific Aim III is now in preparation.**

Specific Aim IV.

The inflammatory response in the brain following injury is thought to contribute to the neurodegenerative process typical of PD and Alzheimer's disease.. Microglial activation and proinflammatory and cytokine up-regulation (Gonzales-Scarano et al, 1999; Vila et al, 2001) are all part of the inflammatory response. Since we are investigating the contribution of inflammation to the neurodegenerative process, it is necessary to examine the components which contribute to this process. Two such components are the cyclooxygenase (COX) enzymes, COX-1 and COX-2. The COX enzymes convert arachidonic acid to prostaglandin PGH_2, the precurser of the prostaglandin PGE_2 and several other prostanoids (O'Banion, 1999). In a earlier publication in the Proceedings of the National Academy of Science entitled **Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration**, we demonstrated that COX-2 rather than COX-1 is up-regulated in SNpc dopaminergic neurons in PD and in the MPTP mouse model of PD. We also noted that this COX-2 up-regulation following MPTP administration and in PD brains occurs through a Jun kinase (JNK)/c-Jun-dependent mechanism Furthermore, we also show that COX-2 inhibition and ablation in the MPTP mouse model of PD prevents the formation of dopamine-o-quinone and attenuates dopaminergic neuron death in the SNpc of treated mice. The inhibition of COX-2 was identified as a possible therapeutic target for drug therapy in PD. **See attached manuscript 3.** In continuing our studies on COX-2, we investigated whether COX-2 is also involved in apoptotic cell death in SNpc dopaminergic neurons as apoptosis is the poposed form of cell death in PD. Thus, following the chronic administration of MPTP (30 mg/kg i .p., daily x 5 days) to COX-2 deficient mice and their non-knockout littermates, we noted that ablation of COX-2 attenuated significantly, the number of apoptotic cells seen in te SNpc of MPTP-

treated mice. At 21 days after the cessation of MPTP treatment, the number of surviving SNpc dopaminergic neurons were 75% of the saline-treated mice in the COX-2 deficient mice These findings indicate that COX-2 may play a role in the activation of the apoptotic molecular machinery that leads to apoptotic DA neuron death in the MPTP mouse model of PD. This work was presented in abstract form at the Society for Neuroscience Meetings in October 2004. **See attached abstract 4**. We will continue studies of this observation and are breeding more COX-1 and COX-2 mice to determine the role of PGE_2 in the production of apoptosis in the SNpc of MPTP-treated mice and of PD brains..

Reportable Outcomes for 2003-2004

1). Core B. The MPTP Facility is operating smoothly. This year, a number of individuals from institutions such as Woods Hole, have visited our facility to see how an MPTP facility should operate. Also, inspection by AALAC and the university's Institutional Animal Care and Use Committee found no deficiencies in this facility. In fact, our facility has been used several times by Columbia University to demonstrate how a satellite facility should operate.
2) Specific Aim III: M40401, a SOD mimetic significantly attenuated MPTP-induced SNpc dopaminergic neuron death as evidenced by an increase in the nymber of surviving neurons and reduced the presence of activated microglia, protein carbonyl and 3-nitrotyrosine. This effect was due to a decrease in the production of the superoxide radical as evidenced by the decrease in hydroethidium fluorescence in the SNpc of MPTP-M40401 treated mice. M40404 and M40435, two inactive isomers of M40401 were ineffective against the damaging effects of MPTP.
3) Specific Aim IV: As a follow-up to our findings in COX-2 deficient mice using our acute schedule of MPTP, we administered chronic MPTP to COX-2 deficient mice and their normal littermates. Findings were presented at the Society for Neuroscience Meeting in November of 2004. We found that the ablation of COX-2 significantly decreased the number of apoptotic cells in the SNpc of mice treated to a chronic schedule of MPTP. These results indicate that COX-2 may have a role in the activation of the apoptotic machinery and may be a good target for therapeutic intervention in the treatment of PD.

Conclusion

PD is now thought to include an inflammatory component and it is this component that we and others think may be contributing to the progressive nature of the disease. This contention is strengthened by the fact that Langston and colleagues (Langston et al, 1999) found an active ongoing inflammation in post mortem tissues from the brains of individuals who had injected street preparations of a synthetic heroin contaminated with MPTP and who exhibited a parkinsonian syndrome. MPTP is selective in its effects in that it kills mainly dopaminergic neurons in the SNpc of the brain in a time-dependent manner that resembles end-stage PD Jackson-Lewis et al, 1995). This makes MPTP an ideal tool with which to study dopaminergic neuron death in the SNpc and to delineate the cellular and molecular mechanisms involved in this process. In earlier studies, we noted that MPTP increased superoxide radical (Przedborski et al, 1992) and nitric oxide (NO) (Liberatore et al, 1999) production. Both compounds are indeed part of the oxidative stress hypothesis of PD (Przedborski and Jackson-Lewis, 2000) and have been found to be present in postmortem tissues from PD brains as well. Thus, we thought it important to determine the sources of

these compounds and to devise therapeutic strategies to lessen their effect in PD and in the MPTP mouse model of PD.

We noted that ramified microglia, barely detectable in the resting state in the normal SNpc, became activated 24 hours after acute MPTP administration and that these cells were the source of the NO due to the presence of inducible nitric oxide synthase (iNOS) up-regulated during the activation of microglia (Liberatore, 1999). Also, microglial activation parallels dopaminergic neuron death in the SNpc of MPTP-treated mice (Liberatore et al, 1999). Our first thought in this project was to block the observed microglial activation to see if we could attenuate the MPTP-evoked SNpc neuron death. To do this, we used minocycline, a second generation tetracycline antibiotic to ascertain a neuroprotective effect, if any in this model. Administration of minocycline to MPTP-treated mice attenuated significantly SNpc dopaminergic neuron death and dramatically reduced the MPTP-induced microglial activation (Wu et al, 2002). Blockade of the up-regulation of iNOS is the mechanism by which minocycline attenuated SNpc dopaminergic neuron death because this compound reduced the amount of NO available for reaction with the superoxide radical. Although this attenuation of neuron death in the SNpc of MPTP-treated mice was independent of minocycline's antibiotic property, the fact that inflammation is a part of infection and that NO plays a role in the infectious process, attests to the reasonableness of this approach. NO is a molecule that can be produced at one site and travel up to 300 microns to another site to interact with any number of other molecules (Radi et al, 2002). In our model, we believe that NO reacts with the superoxide radical to produce peroxynitrite which is the real culprit according to the oxidative stress hypothesis of PD. The decrease in microglial activation while significant with minocycline is not total which led us to believe that other factors are involved in the MPTP-induced inflammatory response.

Since we had noted early on that there was a significant increase in the production of the superoxide radical following MPTP administration (Przedborski et al, 1992), we needed to identify the sources of the superoxide radical production. As demonstrated earlier (Radi et al, 2002;), within the dopaminergic neuron, mitochondria are a source of the superoxide radical. Blockade of the complex I site of the mitochondrial transport chain (METC) by MPP+ (1-methyl-4-phenylpyridinium) the active metabolite of MPTP kicks the superoxide radical out into the cytosol of the dopaminergic neuron. Another source of the superoxide radical are the microglia through the up-regulation of NADPH oxidase. This multimeric enzyme, composed of 4 subunits (GP^{91phox}, p_{22phox}, p^{47phox} and p^{40phox}) is inactive in resting microglia because p^{47phox}, p^{67phox} and p^{40phox} are all present in the cytosol of microglia as a complex and are separated from the transmembrane proteins, GP^{91phox} and p^{22phox} (Babior, 1999). When microglia become activated, p^{47phox} is phosphorylated and the entire complex translocates to the plasma membrane where it assembles with GP^{91phox} and p^{22phox} to form the NADPH oxidase complex. which is now capable of reducing oxygen to the superoxide radical. We used both RT-PCR and immunostaining techniques to demonstrate that the NADPH oxidase complex is not only up-regulated in the SNpc of MPTP-treated mice but peaks at 24-48 hours after MPTP administration. The same tissue sections were then subjected to MAC-1 immunostaining which documented that the cells that exhibited the up-regulation of NADPH oxidase none other than activated microglia. Noticed was the fact that the up-regulation of NADPH oxidase paralleled the up-regulation of the iNOS enzyme and that the source of the

NADPH oxidase was none other that the activated microglia (Wu et al, 2003). Interestingly, the up-regulation of NADPH oxidase was also found in post-mortem tissues from PD brains which fits with our oxidative stress theory of PD (Wu et al,2003). Furthermore, to clarify that MPTP stimulates the generation of the superoxide radical in the SNpc of the treated mice, we used hydroethidium histochemistry and found that 24-48 hours after MPTP administration, there is a significant presence of the superoxide radical in the SNpc of these mice (Wu et al, 2003). Thus, with the sources of the superoxide radical and NO in close proximity with each other, it is befitting to think that these two compounds conspire to injure the dopaminergic neuron in the SNpc following MPTP administration by interacting to produce peroxynitrite, the strong oxidant suggested to cause the death of the dopamine neuron in the MPTP mouse model of PD and in PD itself (Vila and Przedborski, 2003).

Blocking or reducing the presence of the superoxide radical should reduce damage to the dopaminergic neuron in the SNpc and in the MPTP mouse model of PD in relation to the oxidative hypothesis of PD. In the course of metabolism within the DA neuron, the superoxide radical can be produced in several different ways. First of all, DA itself is metabolized by monoamine oxidase (MAO) (Burke et al, 2004), an outer mitochondrial membrane enzyme, which results in a two-electron reduction of oxygen and the production of both the superoxide and the hydroxyl radical In PD, as a result of the loss of the DA neurons, the remaining DA neurons become hyperactive and tend to exhibit increased DA turnover which results in increased ROS production (Przedborski and Jackson-Lewis, 2000). Furthermore, presumably, since L-DOPA therapy increases brain levels of DA. DA can auto-oxidize to increase the ROS load in the brain which includes the superoxide radical (Fahn and Cohen, 1992; Graham, 1978). Also, NADPH oxidase (Wu et al, 2003) and cyclo-oxygenase-2 (COX-2) (Teismann et al, 2003) both inducible enzymes in pathological situations such as PD, generate the superoxide radical. Finally, reduction of complex I of the mitochondrial electron transport chain results in leakage of the superoxide radical (Nicklas et al, 1985) into the cytosol of the neuron. These scenarios represent at least three oxidative stress situations (mitochondrial, cytosolic and inflammatory) in which the superoxide radical is produced. The first line of defense against the superoxide radical are the SOD enzymes. Two of the three SOD enzymes are of concern to PD, the copper-zinc (CuZnSOD; SOD1) form found in extremely high amounts in substantia nigra (SNpc) DA neurons and shown to be unchanged in striatum and in SNpc in PD brains ((Przedborski and Jackson-Lewis, 2000) and the manganese (MnSOD; SOD2) form found in mitochondria within the neuron, known to be inducible in response to ROS production and shown to be increased in both striatum and SNpc in PD brains (Przedborski and Jackson-Lewis, 2000). In this respect, because it is inducible, MnSOD is thought to be neuroprotective as mice lacking this enzyme have a very short lifespan and have mitochondria that do not function properly (Melov et al, 2001). Furthermore, the SNpc of mice overexpressing MnSOD is protected against MPTP-induced degeneration (Klivenyi et al, 1998). Thus, while both isoforms of SOD are necessary for the cell to survive, MnSOD seems to be the more important of the two.

Recently, using molecular modeling systems studies, a stable and active class of SOD mimetics which catalyze the dismutation of the superoxide radical with rates similar to that of native MnSOD has been synthesized. These low molecular weight non-peptidyl compounds, which lack catalase activity, distribute widely in the body, keep their chemical

identity and can be recovered intact in the urine and feces (Salvemini et al, 1999). The parent compound, M40403, does not react with NO, H2O2 or OONO and has been shown to counteract edema, inhibit neutriphil infiltration and the release of such proinflammatory cytokines as TNF-α and IL1-β (Salvemini, 1999). A related compound, M40401, which possesses a catalytic rate constant at least equal to or greater than that of the native SOD enzymes, showed a significant protective effect against the neuropathological and ultrastructural changes effected by bilateral occlusion of the common carotid artery and ischaemia-reperfusion injury in the brain (Cuzzocrea et al, 2001). Both situations generate significant amounts of the superoxide radical thus they throw the brain into an oxidative stress situation, as does MPTP (Przedborski et al, 1992). In the MPTP mouse model of PD, M40401 not only attenuated dopamine neuron death in the SNpc of MPTP-treated mice, but also suppressed superoxide radical formation here by reducing the microglial response to MPTP. In our NADPH studies, we infused Cu/ZnSOD into the striatum to block some of the damaging effects of MPTP. Since SOD is a bulky molecule, it does not cross membranes, thus the protective effect of the Cu/ZnSOD was extracellular and partial in nature as it probably targeted only the superoxide radical produced by the induction of NADPH oxidase. The neuroprotective effect of M40401 on the SNpc dopaminergic neuron tended to be more dramatic as this compound does cross membranes (Salvemini, 1999). Thus, while targeting primarily the superoxide radical produced within mitochondria, it is likely that its effects of M40401 are both intracellular and extracellular because M40401 also reduced the MPTP-induced microglial response which decreased the availability of the superoxide radical for interaction with NO in and around microglia and may have prevented the release of other damaging compounds such as pro-inflammatory cytokines.

In furthering our investigation of the inflammatory process in the MPTP mouse model as it relates to PD, and to simulate a model more akin to PD, we examined the cell death response to a chronic regimen of MPTP in COX-2 knockout mice. Data from our acute model (Teismann et al, 2003) documented that COX-2 enzyme activity and protein levels are significantly higher in MPTP-treated mice than in control mice. In this model, robust COX-2 immunostaining was also noted in both the human and the mouse brain situations and appeared to be confined to the cytosol of dopaminergic neurons (Teismann et al., 2003). Furthermore, inhibition of the COX-2 response to acute MPTP prevented the rise in protein cysteinyldopamine which was shown to occur in mice following the administration of MPTP and no COX-2 inhibitor (Teismann et al, 2003). In the chronic MPTP mouse model, we show that apoptosis, the suggested form of cell death in PD, was significantly less in COX-2 knockout mice than in their non-engineered littermates. This demonstration is better related to PD and suggests that the COX-2 enzyme may play a role in the activation of the molecular pathways instrumental in producing the apoptotic form of cell death. Whether this chronic MPTP model will give the same results as the acute MPTP model remains to be tested.

In conclusion, these studies have demonstrated that at least two components of the central nervous system contribute to the MPTP neurotoxic process in the SNpc of the mouse brain. Activated microglia contribute directly to this toxicity through NADPH oxidase which mediates superoxide radical formation and through the induction of nitric oxide synthase. The neuronal contribution to this effort is most likely through the overproduction of the

superoxide radical through the METC via COX-2. We have also demonstrated that blocking the induction of these enzymes by agents such as M40401 and minocycline significantly increases the number of surviving SNpc dopaminergic neurons by decreasing oxidative stress. Thus, both NADPH oxidase and iNOS may represent useful targets for intervention in the inflammatory cascade of events thought to contribute to the death of SNpc dopaminergic neurons in the MPTP mouse model of PD and ultimately in PD itself.

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Appendices (Publications supported by this grant)

Full Papers

Teismann Teismann P, Tieu K, Choi DK, et al. Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. PNAS (USA) (2003) 100: 5473-5478.

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Abstracts

D. Prou, P.Teismann, V.J Jackson-Lewis, M.Vila and S.Przedborski.
Genetic ablation of COX-2 attenuates dopaminergic programmed cell death in experimental parkinsonism. Soc Neurosci Abstract ID number serge 000138470 password 2004 Genetic

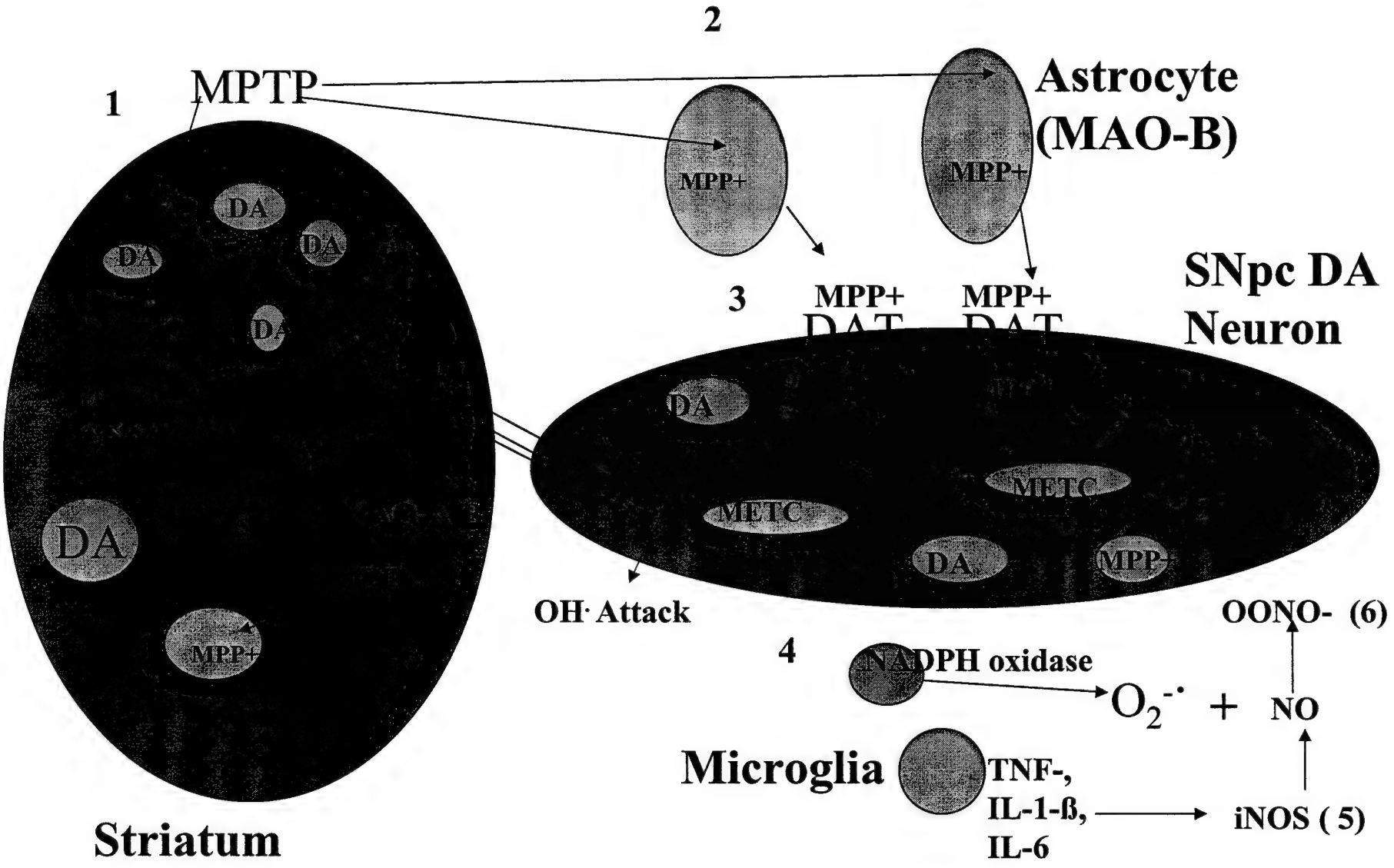

Figure 1. Involvement of Superoxide, Nitric oxide and Dopamine in MPTP Toxicity of theDopamine Neuron.

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Blockade of Microglial Activation Is Neuroprotective in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson Disease

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages the nigrostriatal dopaminergic pathway as seen in Parkinson's disease (PD), a common neurodegenerative disorder with no effective protective treatment. Consistent with a role of glial cells in PD neurodegeneration, here we show that minocycline, an approved tetracycline derivative that inhibits microglial activation independently of its antimicrobial properties, mitigates both the demise of nigrostriatal dopaminergic neurons and the formation of nitrotyrosine produced by MPTP. In addition, we show that minocycline not only prevents MPTP-induced activation of microglia but also the formation of mature interleukin-1β and the activation of NADPH–oxidase and inducible nitric oxide synthase (iNOS), three key microglial-derived cytotoxic mediators. Previously, we demonstrated that ablation of iNOS attenuates MPTP-induced neurotoxicity. Now, we demonstrate that iNOS is not the only microglial-related culprit implicated in MPTP-induced toxicity because mutant iNOS-deficient mice treated with minocycline are more resistant to this neurotoxin than iNOS-deficient mice not treated with minocycline. This study demonstrates that microglial-related inflammatory events play a significant role in the MPTP neurotoxic process and suggests that minocycline may be a valuable neuroprotective agent for the treatment of PD.

Key words: IL-1β; iNOS; minocycline; microglia; MPTP; NADPH–oxidase; neurodegeneration; Parkinson's disease

Parkinson's disease (PD) is a common neurodegenerative disorder whose cardinal clinical features include tremor, slowness of movement, stiffness, and postural instability (Fahn and Przedborski, 2000). These symptoms are primarily attributable to the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the consequent loss of their projecting nerve fibers in the striatum (Hornykiewicz and Kish, 1987; Pakkenberg et al., 1991). Although several approved drugs do alleviate PD symptoms, chronic use of these drugs is often associated with debilitating side effects (Kostic et al., 1991), and none seems to dampen the progression of the disease. So far, the development of effective neuroprotective therapies is impeded by our limited knowledge of the pathogenesis of PD. However, significant insights into the mechanisms by which SNpc dopaminergic neurons may die in PD have been achieved by the use of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which replicates in humans and nonhuman primates a severe and irreversible PD-like syndrome (Przedborski et al., 2000). In several mammalian species, MPTP reproduces most of the biochemical and pathological hallmarks of PD, including the dramatic neurodegeneration of the nigrostriatal dopaminergic pathway (Przedborski et al., 2000).

To elucidate PD pathogenic factors, and thus to develop therapeutic strategies aimed at halting its progression, we revisited the neuropathology of this disease in search of putative culprits. Aside from the dramatic loss of dopaminergic neurons, it appears that the SNpc is also the site of a robust glial reaction in PD and experimental models of PD (Vila et al., 2001b). Although gliosis and especially activated microglia may sometimes be associated with beneficial effects, often gliosis appears to be deleterious (Vila et al., 2001b). For instance, microglial cells, which are resident macrophages in the brain, have the ability to react promptly in response to insults of various natures (Kreutzberg, 1996) in that resting microglia quickly proliferate, become hypertrophic, and increase or express *de novo* a plethora of marker molecules (Banati et al., 1993; Kreutzberg, 1996). The multifunctional nature of activated microglia encompasses the upregulation of cell surface markers such as the macrophage antigen complex-1 (MAC-1), phagocytosis, and the production of cytotoxic molecules, including reactive oxygen species (ROS), nitric oxide (NO), and a variety of proinflammatory cytokines such as interleukin-1β (IL-1β) (Banati et al., 1993; Gehrmann et al., 1995; Hopkins and Rothwell, 1995). Given this, there is little doubt that activated microglia, through the actions of aforementioned factors, can inflict significant damage on neighboring cells.

Minocycline, a semisynthetic second-generation tetracycline, is an antibiotic that possesses superior penetration through the

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brain–blood barrier (Aronson, 1980). Minocycline has emerged as a potent inhibitor of microglial activation (Amin et al., 1996; Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a), an anti-inflammatory property completely separate from its antimicrobial action, and as an effective neuroprotective agent in experimental brain ischemia (Yrjanheikki et al., 1998, 1999), in the R6/2 mouse model of Huntington's disease (Chen et al., 2000), in traumatic brain injury (Sanchez Mejia et al., 2001), and in the 6-hydroxydopamine model of PD (He et al., 2001). In the present study, we report that, in the MPTP mouse model of PD, minocycline (1) mitigates, in a dose-dependent manner, the loss of dopaminergic cell bodies in the SNpc and of nerve terminals in the striatum, (2) reduces the levels of nitrotyrosine, a marker of protein nitrative modification, (3) prevents microglial activation with minimal effects on the astrocytic response, (4) reduces the formation of mature IL-1β and decreases activation of NADPH–oxidase and upregulation of inducible nitric oxide synthase (iNOS), two enzymes implicated in microglial-derived production of ROS and NO, respectively, and (5) protects against MPTP beyond the beneficial effect of iNOS ablation (Liberatore et al., 1999; Dehmer et al., 2000).

MATERIALS AND METHODS

Animals and treatment. All mice used in this study were 8-week-old male C57BL/6 mice from Charles River Laboratories (Wilmington, MA) and iNOS-deficient mice (C57BL/6-NOS2; The Jackson Laboratory, Bar Harbor, ME) and their wild-type littermates weighing 22–25 gm. For MPTP intoxication, mice received four intraperitoneal injections of MPTP-HCl (18 or 16 mg/kg of free base; Sigma, St. Louis, MO) in saline at 2 hr intervals. For minocycline treatment, mice received twice daily (12 hr apart) intraperitoneal injections of varying doses of minocycline-HCl ranging from 1.4 to 45 mg/kg (Sigma) in saline starting 30 min after the first MPTP injection and continuing through 4 additional days after the last injection of MPTP; control mice received saline only. Mice (n = 5–8 per group; saline–saline, saline–minocycline, MPTP–saline, and MPTP–minocycline) were killed at selected time points, and their brains were used for morphological and biochemical analyses. Procedures using laboratory animals were in accordance with the National Institutes of Health guidelines for the use of live animals and were approved by the institutional animal care and use committee of Columbia University. MPTP handling and safety measures were in accordance with our published recommendations (Przedborski et al., 2001b).

Immunoblots. Cytosolic and particulate fractions from selected mouse brain regions were prepared as described previously (Vila et al., 2001a) and used for either one-dimensional Western blot or dot-blot analyses. For Western blots, the following primary antibodies were used: monoclonal anti-p67phox (1:1000; Transduction Laboratories, Lexington, KY), polyclonal anti-calnexin (1:2000; Stressgen, Victoria, British Columbia, Canada). For dot-blot analyses, 25 μg of protein extracts were loaded onto the 0.2 μm nitrocellulose membrane in dot-blot apparatus (Bio-Rad, Hercules, CA), and blots were probed with an affinity-purified polyclonal antibody against nitrotyrosine (1:1000) (Przedborski et al., 2001a) that was preconjugated overnight at 4°C with 1:5000 dilution of horseradish-labeled donkey anti-rabbit IgG. For all blots, bound primary antibody was detected using a horseradish-conjugated antibody against IgG and a chemiluminescent substract (SuperSignal Ultra; Pierce, Rockford, IL). All films were quantified using the NIH Image analysis system.

RNA extraction and reverse transcription-PCR. Total RNA was extracted from midbrain, striatal, and cerebellar samples from all four groups of mice at selected time points and used for reverse transcription-PCR analysis as described previously (Vila et al., 2001a). The primer sequences used in this study were as follows: for mouse MAC-1, 5′-CAG ATC AAC AAT GTG ACC GTA TGG-3′ (forward) and 5′-CAT CAT GTC CTT GTA CTG CCG C-3′ (reverse); for mouse glial fibrillary acidic protein (GFAP), 5′-CAG GCA ATC TGT TAC ACT TG-3′ (forward) and 5′-ATA GCA CCA GGT GCT TGA AC-3′ (reverse); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GTT TCT TAC TCC TTG GAG GCC AT-3′ (forward) and 5′-TGA TGA CAT CAA GAA GGT GGT GAA-3′ (reverse). PCR amplification was performed for 26 cycles for MAC-1 and GFAP and 18 cycles for GADPH. After amplification, products were separated on a 5% PAGE. Gels were dried and exposed overnight to a phosphorimager screen, and then radioactivity was quantified using a computerized analysis system (Bio-Rad PhosphoImager system).

Immunohistochemistry and stereology. Brains were fixed and processed for immunostaining as described previously (Liberatore et al., 1999). Primary antibodies used in this study were as follows: rat anti-MAC-1 (1:200; Serotec, Raleigh, NC), mouse anti-GFAP (1:1000; Boehringer Mannheim, Indianapolis, IN), and a rabbit polyclonal anti-tyrosine hydroxylase (TH) (1:1000; Calbiochem, San Diego, CA). Immunostaining was visualized by using either 3,3′-diaminobenzine (brown) or SG substrate kit (gray blue; Vector Laboratories, Burlingame, CA). Sections were counterstained with thionin.

The total number of TH-positive SNpc neurons was counted in the various groups of animals at 7 d after the last MPTP or saline injection using the optical fractionator method as described previously (Liberatore et al., 1999). This is an unbiased method of cell counting that is not affected by either the volume of reference (SNpc) or the size of the counted elements (neurons). Striatal density of TH immunoreactivity was determined as described previously (Burke et al., 1990).

Assay of NOS catalytic activity. Ventral midbrain NOS activity was assessed by measuring both the calcium-dependent and calcium-independent conversion of [^{3}H]arginine to [^{3}H]citrulline as described previously (Liberatore et al., 1999).

Mature IL-1β measurement. Ventral midbrain content of mature murine IL-1β was done as described using an enzyme-linked immunosorbend assay kit specific for this cytokine (R & D Systems, Minneapolis, MN) (Li et al., 2000).

Measurement of striatal levels of 1-methyl-4-phenylpyridinium. This was done in MPTP–saline and MPTP–minocycline mice killed at 90 min after one intraperitoneal injection of 18 mg/kg MPTP using an HPLC method with ultraviolet detection (wavelength, 295 nm) as described previously (Przedborski et al., 1996).

Synaptosomal 1-methyl-4-phenylpyridinium uptake. Naïve mice were killed, and their striata were dissected out and processed for uptake experiments as described previously (Przedborski et al., 1992). The uptake of [^{3}H]1-methyl-4-phenylpyridinium (MPP^+) was assessed in the absence and presence of minocycline (concentration raging from 1 to 330 μM). The assay was repeated three times, each time using duplicate samples.

Mouse tissue slices and lactate measurement. Striatal slices (300 μm) were prepared and processed as described by Kindt et al. (1987) using 50 μM MPP^+ and varying concentrations of minocycline (0–333 μM). At the end of the incubation (60 min; 37°C), media were collected and used for lactate quantification by enzymatic assay based on the formation of NADH, followed by 340 nm in a spectrophotometer. The assay was repeated three times, each time using duplicate samples.

Statistical analysis. All values are expressed as the mean ± SEM. Differences between means were analyzed using a two-tail Student's t test. Differences among means were analyzed using one-way ANOVA, with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls *post hoc* testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Minocycline attenuates MPTP-induced dopaminergic neurodegeneration

As illustrated in Figure 1*G*, the numbers of SNpc TH-positive neurons varied significantly among the various groups of mice ($F_{(9,71)} = 7.045$; $p < 0.001$). MPTP, 18 mg/kg for four injections over 8 hr, caused more than a 55% reduction in the number of SNpc dopaminergic neuron numbers, as evidenced by TH immunostaining (Fig. 1*C*,*G*). In MPTP-treated mice, minocycline increased significantly the number of surviving SNpc TH-positive neurons in a dose-dependent manner (Fig. 1*D*,*G*). Minocycline at a dose of 1.4 mg/kg twice daily had no effect on MPTP neuro-

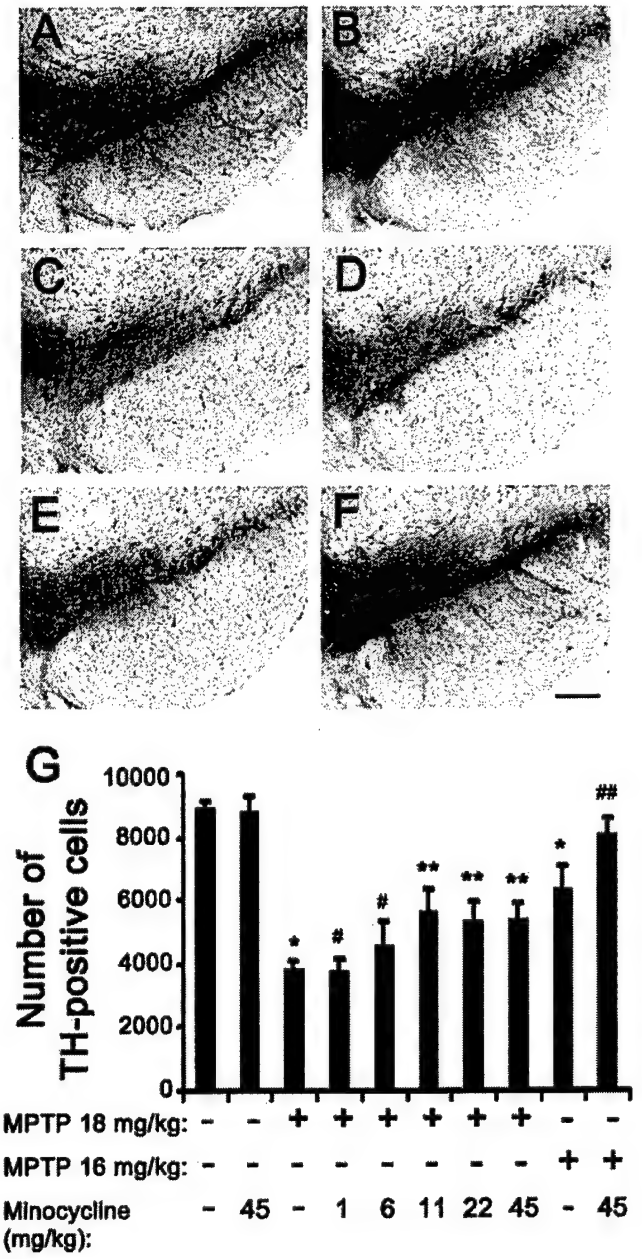

Figure 1. Effect of minocycline on MPTP-induced SNpc dopaminergic neuronal death. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are numerous SNpc TH-positive neurons (*brown*; *A*, *B*). MPTP (18 mg/kg for 4 injections) reduces the number of SNpc TH-positive neurons (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is a noticeable attenuation of SNpc TH-positive neuronal loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is more obvious (*F*). Scale bar, 50 μm. Bar graph shows SNpc TH-positive neuronal counts (*G*) assessed under the various experimental conditions. *Minocycline 1, 6, 11, 22, 45*, Mice injected with minocycline at 1.4, 6.1, 11.3, 22.5, and 45.0 mg/kg twice daily. $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, fewer than control mice but more than MPTP-injected mice. $\#\#p < 0.05$, more than MPTP-injected mice and not different from control mice. Values are means ± SEM (n = 6–8 per group).

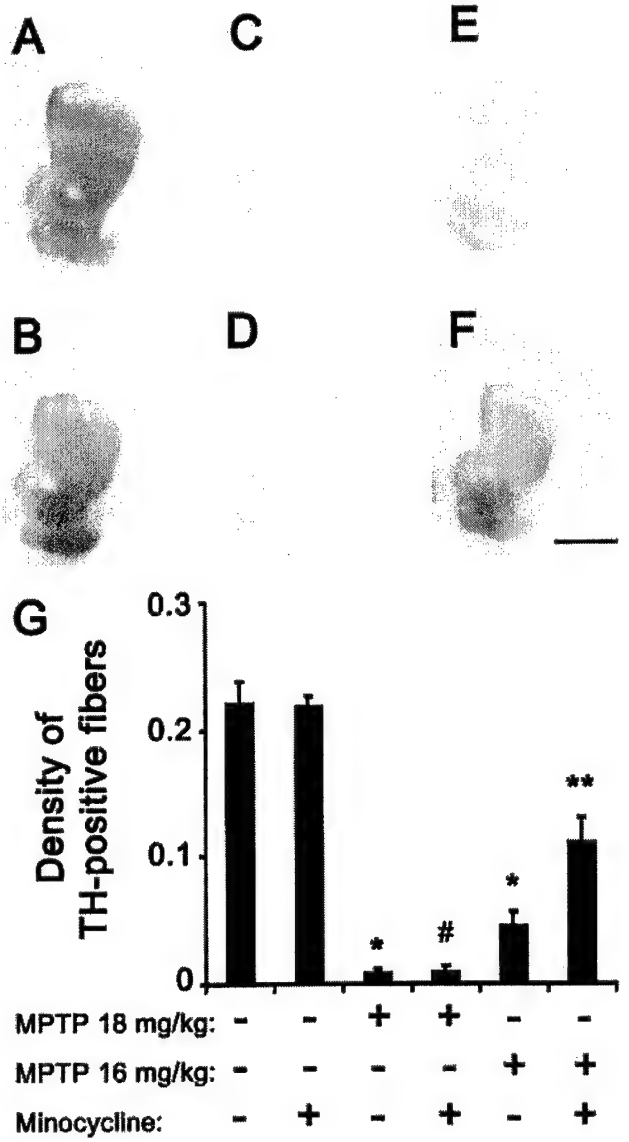

Figure 2. Effect of minocycline on MPTP-induced striatal dopaminergic fiber loss. In saline-injected control mice treated without (*A*) or with (*B*; 45 mg/kg twice daily) minocycline, there are a high density of striatal TH-positive fibers. MPTP (18 mg/kg for 4 injections) reduces the density of striatal TH-positive fibers (*C*) 7 d after the last injection. In mice treated with both MPTP and minocycline, there is also a noticeable striatal TH-positive fiber loss (*D*). At a lower MPTP dosage (16 mg/kg for 4 injections), loss of TH-positive structures is less (*E*) and minocycline protection is obvious (*F*). Scale bar, 1 mm. Bar graph shows striatal TH-positive optical density (*G*) assessed under the various experimental conditions ($F_{(5,33)} = 41.475$; $p < 0.001$). $*p < 0.05$, fewer than saline-injected or minocycline-injected control mice. $\#p > 0.05$, same as MPTP-injected mice. $**p < 0.05$, more than MPTP-injected mice but fewer than control mice. Values are means ± SEM (n = 6–8 per group).

toxicity, whereas at doses of 11.25 mg/kg twice daily and higher, there was significant neuroprotection (Fig. 1*G*). Even at the highest dose tested (45 mg/kg twice daily), minocycline was well tolerated and did not produce any behavioral abnormality. To test whether minocycline could provide complete neuroprotection, we examined another group of mice with less severe SNpc damage by

Table 1. Nitrotyrosine levels (pg/μg protein)

	Saline	Minocycline	MPTP	MPTP–minocycline
Ventral midbrain	16.2 ± 1.3	18.5 ± 1.7	32.2 ± 6.0*	21.8 ± 1.8**
Cerebellum	13.1 ± 0.8	14.0 ± 2.1	13.4 ± 1.0	11.7 ± 1.1

Nitrotyrosine levels are significantly different among groups in the ventral midbrain ($F_{(3,23)} = 4.56$; $p < 0.05$) but not in cerebellum ($F_{(3,23)} = 0.618$; $p > 0.05$). *$p < 0.05$, more than saline-injected and minocycline-injected control mice. **$p < 0.05$, less than MPTP-injected mice but not different from both control groups. Saline, Mice injected with saline; Minocycline, mice injected with minocycline only (45 mg/kg twice daily); MPTP, mice injected with MPTP only (18 mg/kg MPTP for 4 injections in one day); MPTP–minocycline, mice injected with both MPTP and minocycline. Values are means ± SEM (n = 6–8 per group).

Table 2. Striatal MPTP metabolism

MPP^+ level				
Treatment	MPTP only	MPTP–minocycline pretreatment	MPTP–minocycline post-treatment	
μg/gm tissue	6.42 ± 0.92	5.21 ± 0.66	6.52 ± 0.59	
[^{3}H]MPP^+ uptake				
Minocycline (μM)	0	10	100	333
% of control	100	98 ± 3	96 ± 3	82 ± 1
MPP^+-induced lactate				
Minocycline (μM)	0	10	100	333
μM/100 mg protein	74 ± 4	71 ± 6	70 ± 6	67 ± 6

For MPP^+ levels, minocycline (45 mg/kg) was given either 30 min before or after MPTP administration. Values are means ± SEM of either six mice per group (MPP^+ levels) or three independent experiments each performed in duplicate ([^{3}H]MPP^+ uptake and lactate levels). None of the presented values differ significantly ($p > 0.05$) from MPTP only (MPP^+ levels) or from 0 μM minocycline ([^{3}H]MPP^+ uptake and lactate levels).

injecting a lower dose of MPTP (16 mg/kg for four injections). In mice that received MPTP only, this lower regimen reduced numbers of SNpc TH-positive neurons by ~30% compared with controls (Fig. 1*E*,*G*). Minocycline at 45 mg/kg twice daily produced >90% protection against MPTP at 16 mg/kg for four injections (Fig. 1*F*,*G*).

Sparing of SNpc dopaminergic neurons does not always correlate with sparing of their corresponding striatal nerve fibers (Liberatore et al., 1999), which is essential for maintaining dopaminergic neurotransmission. To determine whether minocycline can prevent not only MPTP-induced loss of SNpc neurons but also the loss of striatal dopaminergic fibers, we assessed the density of TH immunoreactivity in striata from the different groups of mice (Fig. 2). Four injections of MPTP at 18 and 16 mg/kg reduced striatal TH immunoreactivity compared with controls by 96 and 79%, respectively (Fig. 2*C*,*E*,*G*). Mice that received minocycline (45 mg/kg twice daily) and four injections of 18 mg/kg MPTP (Fig. 2*D*,*G*) showed no protection of striatal dopaminergic fibers, whereas mice that received the same dose of minocycline and four injections of 16 mg/kg MPTP (Fig. 2*F*,*G*) showed significant sparing of striatal TH-positive fibers. These findings indicate that minocycline protects the nigrostriatal pathway against the effects of the parkinsonian toxin MPTP.

Minocycline decreases MPTP-mediated nitrotyrosine formation

A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage (Przedborski et al., 2000), the extent of which can be evaluated by assessing nitrotyrosine levels (Liberatore et al., 1999; Pennathur et al., 1999). In saline-injected mice, the levels of nitrotyrosine in ventral midbrain were similar between non-minocycline and minocycline-treated animals (Table 1). In MPTP-injected mice (18 mg/kg for four injections), nitrotyrosine levels were significantly increased in ventral midbrain (brain region containing SNpc) and unchanged in cerebellum (brain region unaffected by MPTP) (Table 1). MPTP

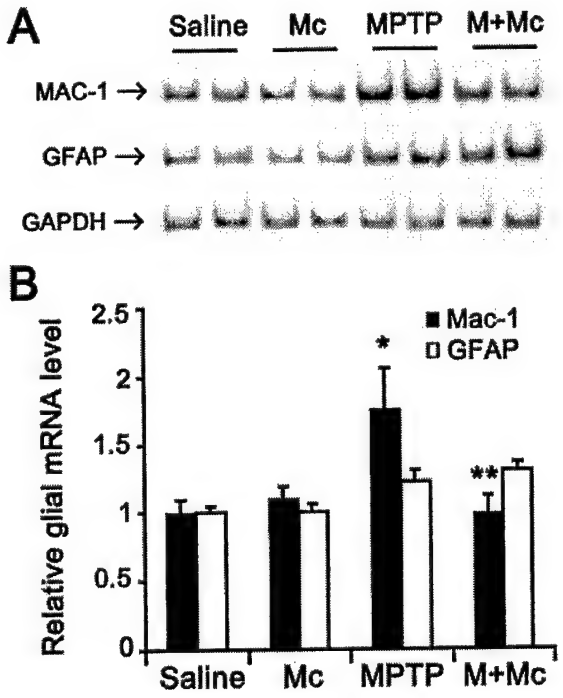

Figure 3. Minocycline prevents MPTP-induced MAC-1 transcription. *A*, *B*, Ventral midbrain MAC-1 mRNA levels but not GFAP mRNA levels are increased by 24 hr after MPTP injection compared with those of saline- or minocycline-injected mice. Minocycline prevents MPTP-induced MAC-1 mRNA increases. MAC-1 and GFAP mRNA values are normalized with GAPDH. Values are mean ± SEM ratios (n = 5–7 mice per group). *Saline*, Saline-treated; *Mc*, minocycline-treated; *MPTP*, MPTP-treated; *M + Mc*, MPTP plus minocycline-treated. *$p < 0.05$, higher than both saline- and minocycline-injected control groups. **$p < 0.05$, lower than MPTP-injected group and not different from both control groups.

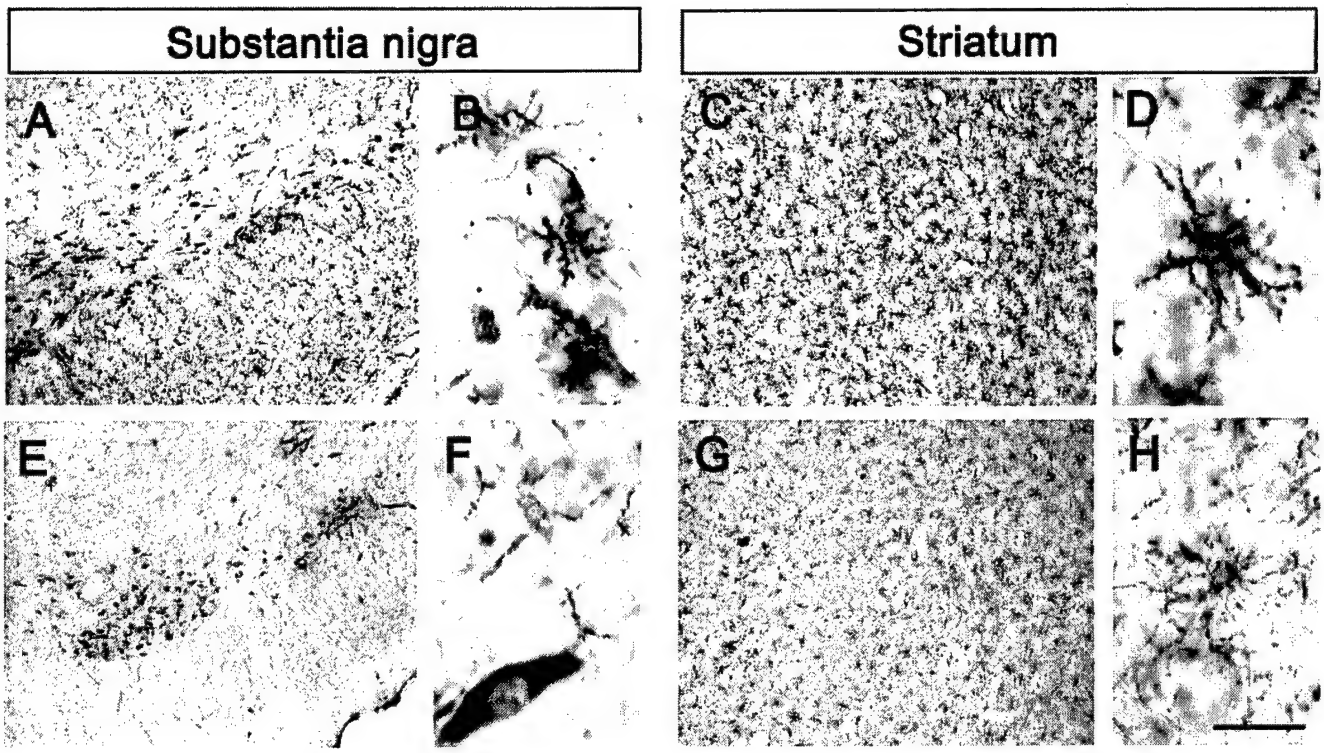

Figure 4. Minocycline prevents MPTP-induced microglia reaction. Microglia cells (*brown*) and TH-positive neurons (*gray blue*) are seen in both SNpc and striatum of all mice. One day after the last MPTP injection, numerous activated microglia (larger cell body, poorly ramified short and thick processes) are seen in SNpc (*A*, *B*) and striatum (*C*, *D*). Mice injected with both MPTP and minocycline show minimal microglial activation in SNpc (*E*) and striatum (*G*); here, microglial cell bodies are small and processes are thin and ramified (*F*, *H*). Scale bar: *A*, *C*, *E*, *G*, 1 mm; *B*, *D*, *F*, *H*, 100 μm.

produced significantly smaller increases in nitrotyrosine levels in ventral midbrains of minocycline (45 mg/kg twice daily)-treated mice than in their non-minocycline-treated counterparts (Table 1). This confirms that minocycline not only attenuates the morphological but also the biochemical impacts of MPTP neurotoxicity.

MPTP metabolism is unaffected by minocycline

The main determining factors of MPTP neurotoxic potency are its conversion in the brain to MPP^+ followed by MPP^+ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski et al., 2000). To ascertain that resistance to the neurotoxic effects of MPTP provided by minocycline was not attributable to alterations in any of these three key MPTP neurotoxic steps, we measured striatal levels of MPP^+ 90 min after injection of 18 mg/kg MPTP, striatal uptake of [^{3}H]MPP^+ into synaptosomes, and striatal MPP^+-induced lactate production, a reliable marker of mitochondrial inhibition (Kindt et al., 1987) (Table 2). These investigations showed that striatal levels of MPP^+ did not differ between MPTP-injected mice that either received or did not receive minocycline (45 mg/kg) 30 min after MPTP administration. In addition, minocycline up to 333 μM (maximal solubilizing concentration) did not affect striatal uptake of [^{3}H]MPP^+ or MPP^+-induced lactate production (Table 2).

Minocycline inhibits MPTP-induced microglial activation

To determine whether neuroprotection by minocycline is associated with inhibition of MPTP-induced glial response, we examined the expression of MAC-1, a specific marker for microglia, and GFAP, a specific marker for astrocytes. As shown in Figure 3*B*, MAC-1 mRNA contents ($F_{(3,23)} = 4.252$; $p < 0.05$), but not GFAP mRNA contents ($F_{(3,18)} = 2.843$; $p > 0.05$), varied significantly among the various group of mice. In saline-injected mice, ventral midbrain expression of MAC-1 and GFAP mRNA was minimal (Fig. 3*A*,*B*). In these animals, only a few faintly immunoreactive resting microglia and astrocytes were observed in SNpc and striatum by immunostaining (data no shown). In MPTP-injected mice (18 mg/kg for four injections) without treatment with minocycline, ventral midbrain expression of MAC-1 mRNA was significantly higher, whereas expression of GFAP mRNA, although also higher, was not significantly increased compared with saline controls (Fig. 3). Morphologically, numerous robustly immunoreactive MAC-1-positive activated microglia were observed 24 hr after the last injection of the toxin (Fig. 4*A–D*). Although GFAP immunostaining appeared somewhat increased at 24 hr after the last MPTP injection (Fig. 5*A*,*B*), the strongest GFAP reaction was noted 7 d after the last injection of MPTP (Fig. 5*C*,*D*). Conversely, in MPTP-injected mice treated with minocycline (45 mg/kg twice daily), ventral midbrain MAC-1 mRNA contents (Fig. 3) and SNpc and striatal immunostaining were similar to those seen in saline-injected mice (Fig. 4*E–H*). In contrast, in MPTP-injected minocycline-treated mice, ventral midbrain GFAP mRNA content (Fig. 3) and SNpc immunostaining (Fig. 5*E*,*F*) were almost as high and as intense as in MPTP-only mice. Staining with Isolectin B-4 (Sigma), another marker for microglia, gave results similar to that of MAC-1 (data not shown).

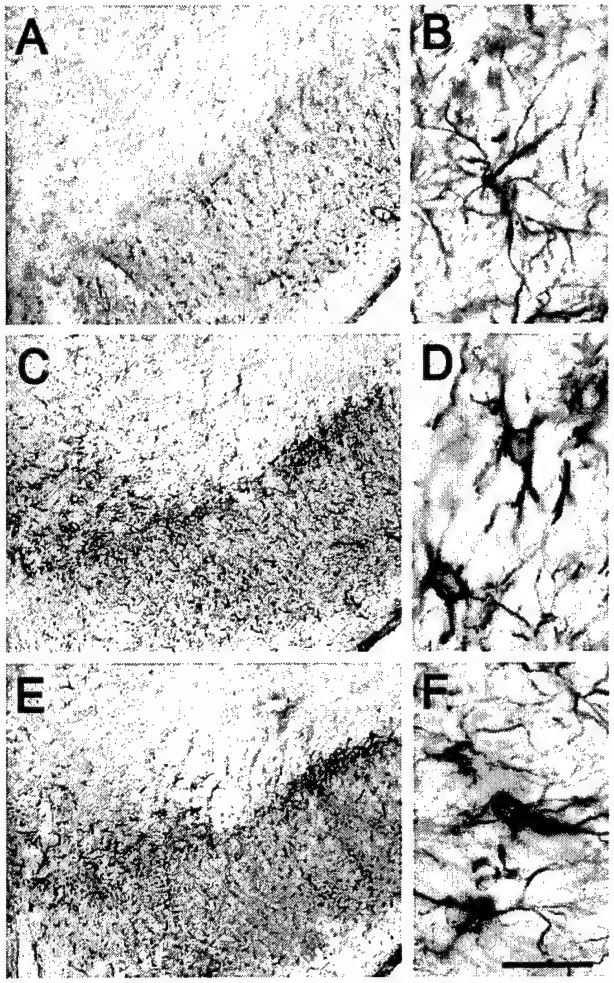

Figure 5. Minocycline does not affect MPTP-induced astrocytic reaction. One day after the last injection of MPTP, there is a mild astrocytic response (*A*, *B*), but 7 d after the last injection of MPTP, it becomes conspicuous (*C*, *D*). Minocycline does not affect the astrocytic response (*E*, *F*) 7 d after MPTP administration. Scale bar: *A*, *C*, *E*, 1 mm; *B*, *D*, *F*, 100 μm.

Minocycline prevents the production of microglial-derived deleterious mediators

Given the effect of minocycline on MPTP-induced microglial activation, we assessed whether the production of known microglial noxious mediators such as IL-1β, ROS, and NO will also be inhibited by minocycline (Fig. 6). The levels of ventral midbrain IL-1β differed significantly among the four group of mice ($F_{(3,21)} = 7.946$; $p < 0.001$) (Fig. 6*A*). Ventral midbrain levels of the proinflammatory cytokine IL-1β in MPTP-injected mice (18 mg/kg for four injections) were significantly increased (Fig. 6*A*). However, MPTP produced significantly smaller increases in IL-1β levels in ventral midbrain of MPTP mice treated with minocycline (45 mg/kg twice daily) (Fig. 6*A*). iNOS activity ($F_{(3,24)} = 9.055$; $p < 0.001$) and the ratio of membrane/total $p67^{phox}$ ($F_{(3,23)} = 4.336$; $p < 0.05$) also varied significantly among the various groups. iNOS and NADPH–oxidase, two prominent enzymes of activated microglia that produce NO and ROS, respectively, exhibited induction patterns similar to those described for IL-1β in that ventral midbrain iNOS activity was increased by 200% (Fig. 6*B*) and NADPH–oxidase activation, evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, was increased by 80% 24 hr after the last injection of MPTP (Fig. 6*C*,*D*). MPTP-induced iNOS activity and NADPH–oxidase were both abolished by minocycline administration (Fig. 6*B–D*).

Minocycline confers resistance to MPTP beyond iNOS ablation

Previously, it has been demonstrated that iNOS ablation attenuates MPTP neurotoxicity (Liberatore et al., 1999; Dehmer et al., 2000). Thus, to demonstrate whether minocycline-mediated blockade of microglial activation protects solely because it inhibits iNOS induction, we compared the effect of MPTP (16 mg/kg for four injections) on the network of striatal dopaminergic nerve fibers between mutant iNOS-deficient mice that received or did not receive minocycline (45 mg/kg twice daily). As shown in Figure 7, MPTP administration reduced by >80% the striatal density of TH-positive fibers both in wild-type and $iNOS^{-/-}$ mice; this is consistent with our previous data that ablation of iNOS protects against MPTP-induced SNpc dopaminergic neuronal loss but not against MPTP-induced striatal dopaminergic fiber destruction (Liberatore et al., 1999). In contrast, striatal TH-positive fiber densities were more than twofold higher in MPTP-treated wild-type and $iNOS^{-/-}$ mice that received minocycline compared with those that did not receive minocycline (Fig. 7). However, there was no difference in the magnitude of the minocycline beneficial effect between MPTP-treated $iNOS^{-/-}$ mice and their MPTP-treated wild-type counterparts (Fig. 7).

DISCUSSION

The main finding of this study is that inhibition of microglial activation by minocycline protects the nigrostriatal dopaminergic pathway against the noxious effects of the parkinsonian toxin MPTP. In mice that received minocycline, MPTP caused significantly less neuronal death in the SNpc, as evidenced by the greater number of TH-positive neurons, compared with those that received MPTP only (Fig. 1). Although less prominent, a similar observation was made for striatal dopaminergic nerve terminals (Fig. 2). The magnitude of resistance to MPTP in mice appears to result from a balance between the dose of minocycline and the dose of MPTP (Fig. 1), with the greatest neuroprotection observed in mice that received >11.25 mg/kg minocycline twice daily and MPTP at 16 mg/kg four times in 1 d and the least neuroprotection in mice that received the regimen of minocycline at 6.1 mg/kg twice daily and MPTP at 18 mg/kg four times in 1 d. In our study, minocycline was given twice daily beginning on the day of MPTP administration and continuing through 4 d thereafter because of its long half-life (>12 hr) and because we showed that, with this MPTP regimen, nigrostriatal degeneration occurs during the first 4 d after the last injection of MPTP (Jackson-Lewis et al., 1995). Therefore, we cannot exclude that greater protection could have been achieved if minocycline had been administered more frequently or for a longer period of time. Also, because we focused our assessment of nigrostriatal neurodegeneration at 7 d after MPTP administration, we cannot exclude with certainty that minocycline had delayed rather than prevented neuronal death. However, in light of what we know about how minocycline presumably mitigates cellular damage in a variety of experimental models (Tikka and Koistinaho, 2001; Tikka et al., 2001a), the aforementioned possibility appears unlikely. In addition, we did not pretreat mice with minocycline

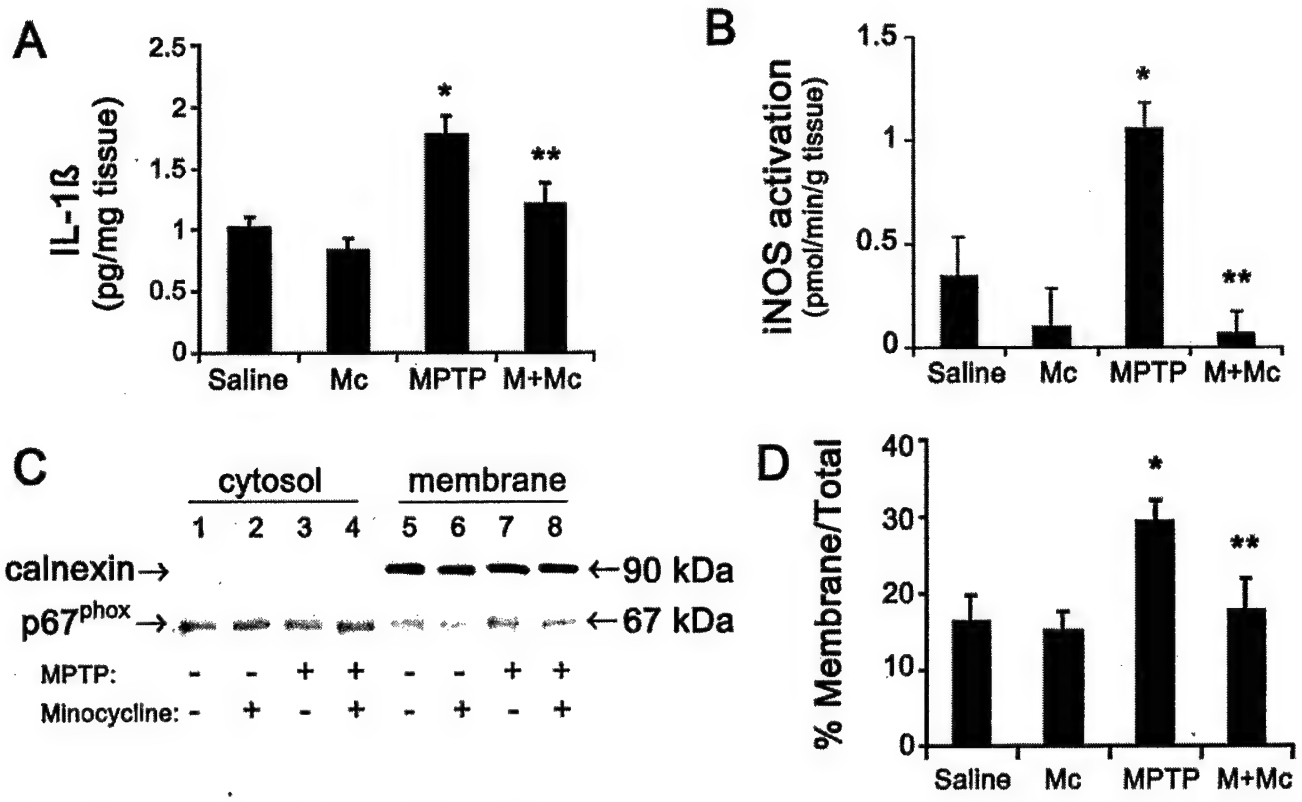

Figure 6. Effects of MPTP and minocycline on microglial-derived deleterious factors IL-1β (*A*), iNOS (*B*), and NADPH–oxidase (*C*, *D*). MPTP (18 mg/kg for 4 injections) increases ventral midbrain mature IL-1β formation, iNOS catalytic activity, and NADPH–oxidase activation, as evidenced by the translocation of its subunit $p67^{phox}$ from the cytosol to the plasma membrane, 1 d after the last injection of MPTP. Minocycline (45 mg/kg twice daily) attenuates MPTP-related effects on mature IL-1β, iNOS, and NADPH–oxidase. *Saline*, Saline-treated; *Mc*, minocycline-treated; *M*, MPTP-treated; *M+Mc*, MPTP plus minocycline-treated. *$p < 0.05$, more than saline-injected or minocycline-injected control mice. **$p < 0.05$, less than MPTP-injected mice but not different from both control groups. Values are means ± SEM (n = 5–8 mice per group).

because we found that administration of minocycline before MPTP injection reduces striatal MPP^+ levels by 20% (Table 2), which could complicate the interpretation of minocycline neuroprotection. Indeed, it is established that striatal contents of MPP^+ correlate linearly with magnitudes of MPTP toxicity (Giovanni et al., 1991). Thus, to avoid this potential confounding factor in our study, all mice were injected first with MPTP and then with minocycline, which we found not to affect striatal MPP^+ levels (Table 2). Along this line, it is also worth mentioning that minocycline, as used here, not only failed to alter MPP^+ levels but also failed to interfere with other key aspects of MPTP metabolism (Przedborski et al., 2000), such as entry of MPP^+ into dopaminergic neurons and inhibition of mitochondrial respiration at concentrations as high as 333 μM (Table 2).

Nitrotyrosine is a fingerprint of NO-derived modification of protein and has been documented as one of the main markers of oxidative damage mediated by MPTP (Schulz et al., 1995; Ara et al., 1998; Liberatore et al., 1999; Pennathur et al., 1999; Przedborski et al., 2001a). Consistent with our previous studies (Liberatore et al., 1999; Pennathur et al., 1999), nitrotyrosine levels increased substantially in brain regions affected by MPTP, such as ventral midbrain, but not in brain regions unaffected by MPTP, such as cerebellum (Table 1). As with the loss of SNpc neurons and striatal fibers, minocycline dramatically attenuated ventral midbrain increases in nitrotyrosine levels (Table 1). Collectively, our data demonstrate that minocycline protects against morphological as well as biochemical abnormalities that arise

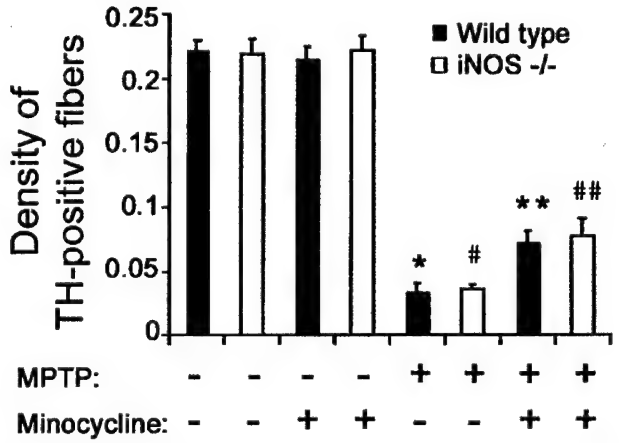

Figure 7. Minocycline attenuates MPTP-induced striatal damage by inhibiting microglia but not just by inhibiting iNOS. The optical density of striatal TH-positive fibers varied significantly among the various groups ($F_{(7,47)} = 83.576$; $p < 0.001$). *Minocycline*, Mice injected with minocycline 45 mg/kg twice daily. MPTP, Mice injected with MPTP (4 injections of 16 mg/kg). *$p < 0.05$, fewer than saline-injected or minocycline-injected control mice. #$p < 0.05$, fewer than control mice but no different than wild-type mice injected with MPTP. **$p < 0.05$, fewer than control but more than MPTP-injected mice. # #$p < 0.05$, more than MPTP-injected mice but no different from wild-type mice injected with both MPTP and minocycline.

from MPTP insult. That said, we now need to consider the nature of the mechanism underlying the beneficial effects of minocycline on MPTP neurotoxicity.

Previously, we demonstrated that, aside from a dramatic loss of dopaminergic neurons, gliosis is a striking neuropathological feature in the SNpc and the striatum in the MPTP mouse model as in PD (Liberatore et al., 1999). However, activated microglia appear in the SNpc earlier than reactive astrocytes (Liberatore et al., 1999) and at a time when only minimal neuronal death occurs (Jackson-Lewis et al., 1995). This supports the contention that the microglial response to MPTP arises early enough in the neurodegenerative process to contribute to the demise of SNpc dopaminergic neurons. Consistent with this is the demonstration that direct injection of the known microglial activator lipopolysaccharide into the rat SNpc causes a strong microglial response associated with significant dopaminergic neuronal death (Castano et al., 1998; Herrera et al., 2000; Kim et al., 2000). Given these data, the key to the minocycline neuroprotective effect in the MPTP mouse model may lie in the second main finding of our study, which is that minocycline prevented MPTP-induced microglial response in both the SNpc and the striatum (Figs. 3, 4). In contrast, minocycline did not alter MPTP-related astrocytic response (Fig. 5). These results suggest that minocycline acts on microglia specifically and not on all components of gliosis. Our data also support the view that reduction of MPTP-related microglial response seen after minocycline administration is not secondary to the attenuation of neuronal loss but rather the reverse. This interpretation does not rule out, however, that at least some of the neuroprotection of minocycline against MPTP is attributable to a direct action on neurons as suggested previously (Tikka et al., 2001b).

Inhibition of microglial activation using minocycline has also been demonstrated *in vitro* (Tikka et al., 2001b) and in other experimental models of acute and chronic brain insults (Yrjanheikki et al., 1998, 1999; Tikka and Koistinaho, 2001; Tikka et al., 2001a) and results, presumably, from the blockade of p38 mitogen-activated protein kinase (Tikka et al., 2001a). It is believed that activated microglia exerts cytotoxic effects in the brain through two very different and yet complementary processes (Banati et al., 1993). First, they can act as phagocytes, which involve direct cell-to-cell contact. Second, they are capable of releasing a large variety of potentially noxious substances (Banati et al., 1993). Consistent with the notion that minocycline inhibits the ability of microglia to respond to injury, we show that minocycline not only prevents the microglial morphological response to MPTP but also the microglial production of cytotoxic mediators such as IL-1β and the induction of critical ROS- and NO-producing enzymes such as NADPH–oxidase and iNOS (Fig. 6). Although we did not test this, it is quite relevant to mention that minocycline may also prevent the induction of cyclooxygenase-2, a key enzyme in the production of potent proinflammatory prostanoids, either directly or indirectly via the blockade of IL-1β formation (Yrjanheikki et al., 1999). Little is known about the actual role of IL-1β in either MPTP or PD neurodegenerative process, except that IL-1β immunoreactivity is found in glial cells from postmortem PD SNpc samples (Hunot et al., 1999) and that blockade of interleukin converting enzyme, the known activator of IL-1β, attenuates MPTP-induced neurodegeneration in mice (Klevenyi et al., 1999). As for ROS, oxidative stress is a prominent pathogenic hypothesis in both MPTP and PD (Przedborski and Jackson-Lewis, 2000). However, many of the microglial-derived ROS, such as superoxide, cannot readily transverse cellular membranes (Halliwell and Gutteridge, 1991), making it unlikely that these extracellular reactive species gain access to dopaminergic neurons and trigger intraneuronal toxic events. Alternatively, superoxide can react with NO in the extracellular space to form the highly reactive tissue-damaging species peroxynitrite, which can cross the cell membrane and injure neurons. Therefore, microglial-derived superoxide, by contributing to peroxynitrite formation, may be significant in this model. As for NO in both MPTP and PD, the pivotal pathogenic role for microglial-derived NO is supported by the demonstration that ablation of iNOS attenuates SNpc dopaminergic neuronal death (Liberatore et al., 1999; Dehmer et al., 2000) and the production of ventral midbrain nitrotyrosine after MPTP administration (Liberatore et al., 1999). In this context, it is worth mentioning that minocycline, which protects in global brain ischemia (Yrjanheikki et al., 1998) and in a mouse model of Huntington's disease (Chen et al., 2000), appears to do so by abating iNOS expression and activity. Remarkably, iNOS ablation does protect SNpc neurons from MPTP toxicity but does not protect striatal nerve terminals and does not prevent microglial activation (Liberatore et al., 1999). This is in striking contrast to the effect of minocycline treatment, which protects both dopaminergic cell bodies and nerve fibers and inhibits the entire microglial response. This strongly suggests that microglial-associated deleterious factors other than iNOS are involved in the demise of the nigrostriatal pathway in the MPTP mouse model of PD and possibly in PD itself. Consistent with this interpretation are our data in iNOS $^{-/-}$ mice (Fig. 7), which show that minocycline protects striatal dopaminergic fibers regardless of the presence or absence of iNOS expression. Therefore, our study provides strong support to the idea that activated microglia are important contributors to the overall demise of SNpc dopaminergic neurons in the MPTP mouse model of PD and, possibility, in PD itself. It also suggests that therapeutic interventions aimed at preventing the loss of striatal dopaminergic fibers, which is essential to maintaining dopaminergic neurotransmission, must target microglial-derived factors other than iNOS.

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Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration

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Parkinson's disease (PD) is a neurodegenerative disorder of uncertain pathogenesis characterized by the loss of the nigrostriatal dopaminergic neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Increased expression of cyclooxygenase type 2 (COX-2) and production of prostaglandin E_2 have been implicated in neurodegeneration in several pathological settings. Here we show that COX-2, the rate-limiting enzyme in prostaglandin E_2 synthesis, is up-regulated in brain dopaminergic neurons of both PD and MPTP mice. COX-2 induction occurs through a JNK/c-Jun-dependent mechanism after MPTP administration. We demonstrate that targeting COX-2 does not protect against MPTP-induced dopaminergic neurodegeneration by mitigating inflammation. Instead, we provide evidence that COX-2 inhibition prevents the formation of the oxidant species dopamine-quinone, which has been implicated in the pathogenesis of PD. This study supports a critical role for COX-2 in both the pathogenesis and selectivity of the PD neurodegenerative process. Because of the safety record of the COX-2 inhibitors, and their ability to penetrate the blood–brain barrier, these drugs may be therapies for PD.

Parkinson's disease (PD) is a common neurodegenerative disease characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (1). Its main neuropathological feature is the loss of the nigrostriatal dopamine-containing neurons, whose cell bodies are in the substantia nigra pars compacta (SNpc) and nerve terminals in the striatum (2). Except for a handful of inherited cases related to known gene defects, PD is a sporadic condition of unknown pathogenesis (1).

Epidemiological studies suggest that inflammation increases the risk of developing a neurodegenerative condition such as Alzheimer's disease (3). In keeping with this suggestion, inflammatory processes associated with increased expression of the enzyme cyclooxygenase type 2 (COX-2) and elevated levels of prostaglandin E_2 (PGE_2) have been implicated in the cascade of deleterious events leading to neurodegeneration in a variety of pathological settings (4–6). COX converts arachidonic acid to PGH_2, the precursor of PGE_2 and several other prostanoids, and exists in eukaryotic cells in two main isoforms: COX-1, which is constitutively expressed in many cell types; and COX-2, which is normally not present in most cells, but whose expression can readily be induced in inflamed tissues (7). Although both isoforms synthesize PGH_2, COX-1 is primarily involved in the production of prostanoids relevant to physiological processes, whereas COX-2 is mainly responsible for the production of prostanoids linked to pathological events (7).

In this study, we asked whether PD is associated with COX-2 up-regulation, and, if so, whether COX-2 expression contributes to the PD neurodegenerative process. We found that COX-2 expression is induced specifically within SNpc dopaminergic neurons in postmortem PD specimens and in the 1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine (MPTP) mouse model of PD during the destruction of the nigrostriatal pathway. We also show that COX-2 induction occurs through a JNK/c-Jun-dependent mechanism and that COX-2 ablation and inhibition attenuate MPTP-induced nigrostriatal dopaminergic neurodegeneration, not by curtailing inflammation, but possibly by mitigating oxidative damage. These findings provide compelling evidence that COX-2 is involved in the pathogenesis of PD and suggest a potential mechanism for the selectivity of neuronal loss in this disease.

Materials and Methods

Animals and Treatments. Wild-type mice were 8-week-old C57/BL/6 specimens (Charles River Breeding Laboratories). *Ptgs1*$^{-/-}$ mice deficient in COX-1 (B6;129P2-Ptgs1^{tm1}), *Ptgs2*$^{-/-}$ mice deficient in COX-2 (B6;129P2-Ptgs2^{tm1}), and their respective wild-type littermates were obtained from Taconic Farms. Genotyping was performed by PCR (8). For each study, 4–10 mice per group received four i.p. injections of MPTP·HCl (20 mg/kg free base; Sigma) dissolved in saline, 2 h apart in one day, and were killed at selected times ranging from 0 to 7 days after the last injection. Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (9). Rofecoxib (12.5–50 mg per kg per day; a gift from Merck Frosst Labs, Pointe Claire, PQ, Canada) was given to mice by gavage for 5 days before and after MPTP-injection. Control mice received vehicle only. This regimen was well tolerated and yielded 0.40 ± 0.06 ng of rofecoxib per mg of tissue (mean ± SEM for five mice) 2 h after the last gavage (measurements were kindly performed by Pauline Luk from Merck Frosst by HPLC with UV detection). Rofecoxib inhibited MPTP-induced PGE_2 production in a dose-dependent manner and did not affect striatal 1-methyl-4-phenylpyridinium (MPP^+) levels in mice (see Tables 2 and 3, which are published as supporting information on the PNAS web site, www.pnas.org). JNK pathway inhibitor CEP-11004 (1 mg/kg; gift from Cephalon, West Chester, PA) was given to mice by s.c. injections 1 day before and 6 days after MPTP-injection as described (10); CEP-11004 did not affect striatal MPP^+ levels in mice (see Table 3). Control mice received the vehicle only. This protocol was in accordance with National Institutes of Health guidelines for the use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University.

RNA Extraction and RT-PCR. Total RNA was extracted from selected mouse brain regions as described (11). The primer sequences for COX-1, COX-2, IL-1β-converting enzyme (ICE), the 91-kDa subunit of NADPH oxidase (gp91), macrophage antigen complex-1 (MAC-1), inducible nitric oxide synthase (iNOS), and GAPDH can be found in refs. 4 and 11. All products were quantified by a phosphorimager (Bio-Rad) or a FluorChem 8800 digital image system (Alpha Innotech, San Leandro, CA).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PG, prostaglandin; COX, cyclooxygenase; SNpc, substantia nigra pars compacta; MPP^+, 1-methyl-4-phenylpyridinium; MAC-1, macrophage antigen complex-1; TH, tyrosine hydroxylase; ICE, IL-1β converting enzyme; iNOS, inducible NO synthase.

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Immunoblots. Mouse and human brain protein extracts were prepared as described (4); for phosphorylated c-Jun analysis, isolating mixture also contained 50 mM NaF and 1 mM Na_3VO_4. Western blot analyses were performed as described (4). Primary antibodies used were as follows: COX-2 (1:250; Transduction Laboratories, Lexington, KY), COX-1 (1:250; Santa Cruz Biotechnology), phosphorylated c-Jun (1:200; Cell Signaling, Beverly, MA), total c-Jun (1:200; Santa Cruz Biotechnology), or β-actin (1:10,000; Sigma). A horseradish-peroxidase-conjugated secondary antibody (1:500–1:25,000; Amersham Pharmacia) and a chemiluminescent substrate (SuperSignal Ultra; Pierce) were used for detection. Bands were quantified by using a FluorChem 8800 digital image system (Alpha Innotech).

PGE_2 Tissue Content. PGE_2 content was assessed in mouse and human tissues by a commercially available high sensitivity chemiluminescence enzyme immunoassay (EIA) kit (4) from Cayman Chemical, Ann Arbor, MI, according to the manufacturer's instructions.

COX-2, Tyrosine Hydroxylase (TH), Glial Fibrillary Acidic Protein (GFAP), and MAC-1 Immunohistochemistry. These were all performed according to our standard protocol for single or double immunostaining (11). Primary antibodies were COX-1 (1:100; Santa Cruz Biotechnology), COX-2 (1:250; gift from W. L. Smith, Michigan State University, East Lansing), TH (1:500; Chemicon), GFAP (1:500; Chemicon), and MAC-1 (1:1,000; Serotec). Immunostaining was visualized by 3,3′-diaminobenzidine with or without nickel enhancement or by fluorescein and Texas red (Vector Laboratories) and was examined by either regular light or confocal microscopy.

TH immunostaining was carried out on striatal and midbrain sections (11) and the TH- and Nissl-stained SNpc neurons were counted by stereology using the optical fractionator method described (11). The striatal density of TH immunoreactivity was determined as described (11).

Measurement of Protein-Bound 5-Cysteinyl-dopamine. Quantification of protein-bound 5-cysteinyl-dopamine was achieved by HPLC with electrochemical detection (12) using mouse brain extracts at 2 and 4 days after MPTP injections.

MPTP Metabolism. Striatal MPP^+ levels were determined by HPLC-UV detection (wavelength, 295 nm; ref. 11) 90 min after the fourth i.p. injection of 20 mg/kg MPTP. Synaptosomal uptake of [^{3}H]MPP^+ was performed as before (11) in *Ptgs2*$^{+/+}$ and *Ptgs2*$^{-/-}$ littermates.

Human Samples. Human samples were obtained from the Parkinson brain bank at Columbia University. Selected PD and controls samples were matched for age at death and interval from death to tissue processing (see *Supporting Text*, which is published as supporting information on the PNAS web site, for details).

Statistical Analysis. All values are expressed as the mean ± SEM. Differences among means were analyzed by using one- or two-way ANOVA with time, treatment, or genotype as the independent factor. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls post hoc testing. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

MPTP Induces COX-2 Expression and Activity in Mouse Ventral Midbrain. To determine whether the expression of COX isoforms is affected during the nigrostriatal neurodegeneration, we assessed the contents of COX-1 and COX-2 mRNA and protein in ventral midbrains (the brain region that contains the SNpc) of saline- and MPTP-injected mice, at different time points. Ventral midbrain COX-1 mRNA and protein were detected in saline-treated mice and their contents were not significantly changed by MPTP administration (Fig. 1 *A*, *B*, *D*, and *E*); there was a decrease of COX-1 mRNA (but not of protein) at 2 and 4 days after MPTP administration, suggesting a transient reduction in COX-1 transcription because of the toxic insult. In contrast, ventral midbrain COX-2 mRNA and protein were almost undetectable in saline-treated mice (Fig. 1 *A*, *C*, *D*, and *F*), but were detected in MPTP-treated mice at 24 h after injections and thereafter (Fig. 1 *A*, *C*, *D*, and *F*). To determine whether MPTP-related COX-2 up-regulation paralleled an increase of its enzymatic activity, we quantified tissue contents of PGE_2. Ventral midbrain PGE_2 is detectable in saline-injected mice, and, as shown by the use of *Ptgs2*$^{-/-}$ and *Ptgs1*$^{-/-}$ mice, derives primarily from COX-1 (see Table 2). Ventral midbrain PGE_2 contents rose during MPTP neurotoxicity, coincidentally to the changes in COX-2 expression (Fig. 1*G*). Although whole-tissue PGE_2 deriving from COX-2 almost doubles after MPTP, ≈65% still originates from COX-1 (see Table 2). Unlike in ventral midbrain, levels of COX-2 mRNA, proteins, and catalytic activity in cerebellum (brain region unaffected by MPTP) and striatum were unaffected by MPTP administration (data not shown). Thus, COX-2, but not COX-1, is up-regulated in the MPTP mouse model.

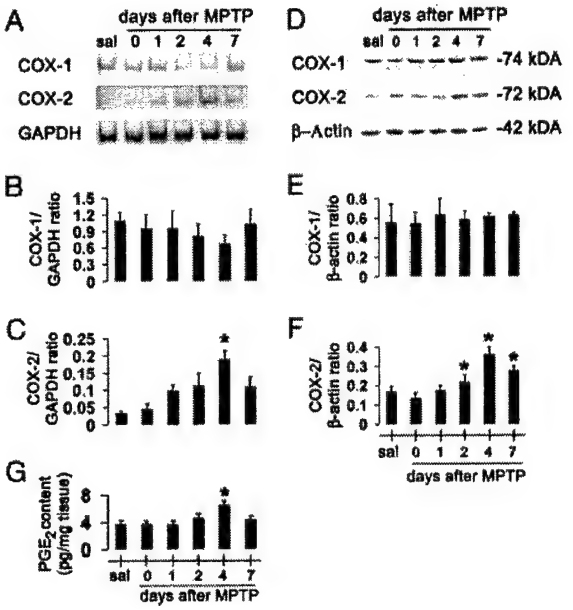

Fig. 1. Ventral midbrain COX-1 and COX-2 mRNA and protein expression after MPTP. COX-2 mRNA levels are increased by 4 days after MPTP injection (*A*) compared with controls (*C*), and almost return to basal levels by 7 days. COX-2 protein contents are minimal in saline-injected mice (sal) (*D*) but rise in a time-dependent manner after MPTP injection (*F*). COX-1 expression is not altered by MPTP (*A*, *B*, *D*, and *E*). Ventral midbrain PGE_2 levels are also increased 4 days after MPTP (*G*). Data are mean ± SEM for four to six mice per group. *, $P < 0.05$, compared with saline (Newman–Keuls post hoc test).

COX-2-Specific Induction in SNpc Dopaminergic Neurons After MPTP Administration. To elucidate the cellular origin of COX-2 up-regulation in the ventral midbrain of MPTP-treated mice, we performed immunohistochemistry. In saline controls, faint COX-2 immunoreactivity was seen in the neuropil (Fig. 2 *A* and *B*). In MPTP-treated mice, at 2 and 4 days after the last injection, ventral midbrain COX-2 immunostaining of the neuropil was increased and several COX-2-positive cells with a neuronal morphology were seen in the SNpc (Fig. 2 *C* and *D*). COX-2-positive neurons showed immunoreactivity over the cytoplasmic and nuclear areas (Fig. 2*D*), which is consistent with the known subcellular localization of this

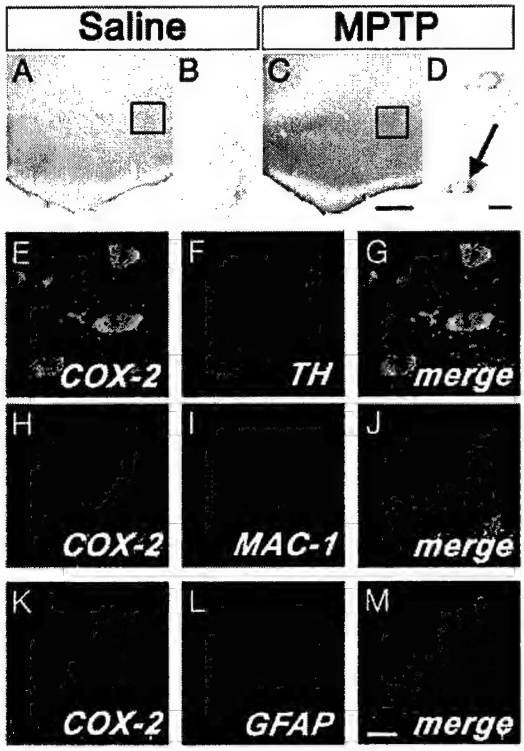

Fig. 2. Ventral midbrain illustration of COX-2 immunolocalization. No COX-2-positive cells are seen in saline-injected mice (*A* and enlarged *Inset* from *A* in *B*). Conversely, COX-2-positive cells are abundant after MPTP (*C* and enlarged *Inset* from *C* in *D*, arrow). Double immunofluorescence confirms that COX-2 (green) is highly expressed in TH-positive neurons (red; *E–G*) and not in MAC-1-positive cells (*H-J*; red) or GFAP-positive cells (*K–M*; red). [Scale bars, 250 μm (*A* and *C*), 10 μm (*B* and *D–G*), and 20 μm (*H–M*).]

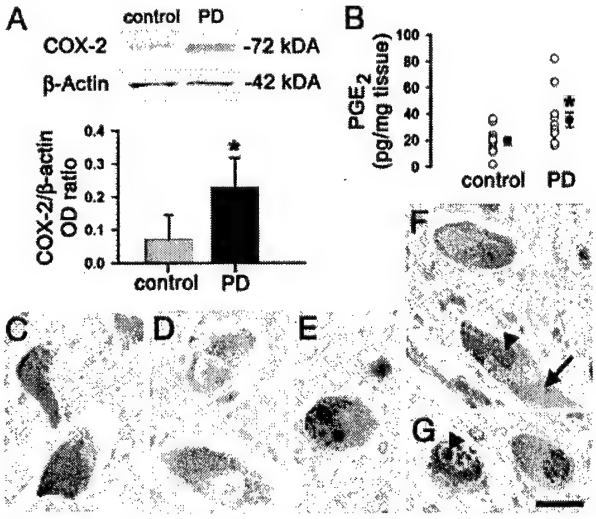

Fig. 3. Ventral midbrain COX-2 expression is minimal in normal human specimens but is increased 3-fold in PD samples (*A*). Ventral midbrain PGE_2 levels are also increased in PD (*B*). COX-2 (blue) is not detected in neuromelanized (brown) dopaminergic neurons in controls (*C* and *D*) but is well detected in PD (*E–G*). COX-2 immunostaining (*F*; *arrow*) is visible in cells with neuromelanin (*F*; arrowhead). COX-2 immunostaining is found in the core of a Lewy body (*G*; arrowhead). Data are mean ± SEM for 3–6 samples for COX-2 protein and 11 samples for PGE_2 assessment. *, $P < 0.05$, compared with normal controls (Newman–Keuls posthoc test). (Scale bar, 25 μm.)

enzyme (13). By double immunofluorescence, we found that ventral midbrain COX-2-positive cells were indeed neurons, among which almost all were dopaminergic (Fig. 2 *E–G*). COX-2 immunofluorescence did not colocalize with the microglial marker MAC-1 (Fig. 2 *H–J*), or with the astrocytic marker GFAP (Fig. 2 *K–M*). No difference in COX-2 immunoreactivity was observed in the striatum between saline- and MPTP-treated mice (data not shown). These data demonstrate that COX-2 is primarily up-regulated in ventral midbrain dopaminergic neurons during MPTP neurotoxicity.

COX-2 Up-Regulation in Postmortem Ventral Midbrain Samples from PD. To assess whether the changes in COX-2 seen after MPTP were present in PD, we assessed COX-2 protein and PGE_2 contents in postmortem SNpc samples. Consistent with the MPTP findings, PD samples had significantly higher contents of COX-2 protein and PGE_2 than normal controls (Fig. 3 *A* and *B*). As in the mice, no significant change in PGE_2 content was seen in the striatum of PD patients (data not shown). Histologically, cellular COX-2 immunoreactivity was not identified in a normal control (Fig. 3 *C* and *D*), but it was in PD midbrain sections, where it was essentially found in SNpc neuromelanized neurons (Fig. 3 *E–G*). Within these dopaminergic neurons, COX-2 immunostaining was seen in cytosol and in the typical intraneuronal proteinacious inclusions, Lewy bodies (Fig. 3*G*). The similarity of the COX-2 alterations between the MPTP mice and the PD postmortem specimens strengthens the value of using this experimental model to study the role of COX-2 in the PD neurodegenerative process.

Ablation of COX-2 Mitigates MPTP-Induced Neurodegeneration. In light of the MPTP- and PD-induced SNpc COX-2 up-regulation, we asked whether this enzyme is implicated in the nigrostriatal degeneration seen in these two pathological situations. Therefore, we compared the effects of MPTP in *Ptgs2*$^{-/-}$, *Ptgs2*$^{+/-}$, and *Ptgs2*$^{+/+}$ mice. Stereological counts of SNpc dopaminergic neurons defined by TH and Nissl staining did not differ among the three genotypes after saline injections (Fig. 4 *A* and *B* and Table 1). SNpc dopaminergic neuron numbers were reduced in all three genotypes after MPTP injections (Fig. 4 *A* and *B* and Table 1). However, in *Ptgs2*$^{-/-}$ mice, and to a lesser extent in *Ptgs2*$^{+/-}$ mice, significantly more TH- and Nissl-stained SNpc neurons survived MPTP administration than in *Ptgs2*$^{+/+}$ mice (Fig. 4*C* and Table 1). In the striatum, the density of TH-positive fibers was decreased to 16% of saline values in MPTP-treated *Ptgs2*$^{+/+}$ and to 21% in *Ptgs2*$^{+/-}$ mice, but only to 63% in *Ptgs2*$^{-/-}$ mice (Table 1). In contrast to the lack of COX-2, the lack of COX-1 did not decrease MPTP neurotoxicity: *Ptgs1*$^{-/-}$ mice [saline = 8,640 ± 725, MPTP = 4,247 ± 554 (mean ± SEM for three to eight mice per group)] and *Ptgs1*$^{+/+}$ littermates (saline = 8,577 ± 334, MPTP = 5,274 ± 147; $P > 0.05$, between MPTP-treated groups, Newman–Keuls posthoc test). Thus, COX-2, but not COX-1, participates in the MPTP neurotoxic process affecting dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

MPTP-Induced Toxicity Requires COX-2 Catalytic Activity. In the absence of catalytic activity, COX-2 can still exert deleterious effects in transfected cells (14). To test whether a similar situation occurs *in vivo* in the demise of dopaminergic neurons mediated by MPTP, nigrostriatal degeneration was assessed in regular mice injected with this neurotoxin and treated with the selective COX-2 inhibitor rofecoxib. The selected regimens of rofecoxib did not cause any distress in the animals (see *Materials and Methods* for details) or any alteration in MPTP metabolism (see below), and afforded meaningful brain accumulation (see *Materials and Methods* for details). At both 25 and 50 mg/kg, rofecoxib completely blocked ventral midbrain COX-2-derived PGE_2 production (see Table 2). In mice injected with MPTP that received either 25 or 50 mg/kg rofecoxib, ≈74% and 88%,

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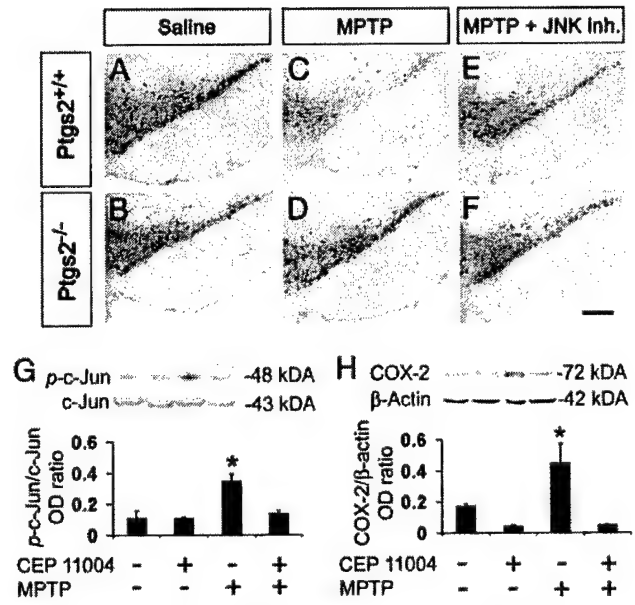

Fig. 4. Effect of COX-2 ablation and JNK pathway inhibition on MPTP-induced neuronal loss. TH-positive neuronal counts are shown in Table 1 and appear comparable between saline-injected *Ptgs2*$^{-/-}$ and *Ptgs2*$^{+/+}$ mice (*A* and *B* and Table 1). SNpc TH-positive neurons are more resistant to MPTP in *Ptgs2*$^{-/-}$ (*D*) than in *Ptgs2*$^{+/+}$ (*C*) mice, 7 days after MPTP injection. CEP-11004 protects *Ptgs2*$^{+/+}$ mice against MPTP neurotoxicity (*E*). Treatment of *Ptgs2*$^{-/-}$ mice with CEP-11004 does not enhance protection against MPTP (*F* and Table 1). (*G*) Ventral midbrain MPTP-induced c-Jun phosphorylation (*p*-c-Jun) inhibition by 1 mg/kg CEP-11004. (*H*) Ventral midbrain MPTP-induced COX-2 up-regulation is also inhibited by 1 mg/kg CEP-11004. Data are mean ± SEM for three to six mice per group. *, $P < 0.05$, compared with the other three groups (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

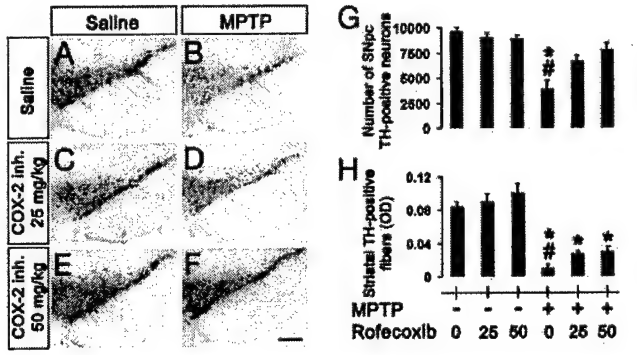

Fig. 5. TH-positive neurons and striatal fibers are more resistant to MPTP in mice treated with rofecoxib (25 or 50 mg/kg p.o.; *D* and *F*) than in mice receiving vehicle (*B*), 7 days after MPTP injection (SNpc neuronal counts are shown in *G* and striatal fiber optical density is shown in *H*). Rofecoxib by itself has no effect on TH-positive neurons (*A*, *C*, and *E*). Data are mean ± SEM for three to six mice per group. *, $P < 0.05$, compared with saline-treated controls; #, $P < 0.05$, compared with rofecoxib-treated MPTP animals (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

respectively, of SNpc TH-positive neurons survived, compared with 41% in mice injected with MPTP only (Fig. 5 *C–G*). Similarly, both doses of rofecoxib attenuated the loss of TH-positive fibers caused by MPTP (Fig. 5*H*) in a dose-dependent manner, although this beneficial effect was less profound than was seen with COX-2 ablation (Table 1). These findings demonstrate how crucial the enzymatic function of COX-2 is to its neurotoxic effects on at least SNpc dopaminergic neurons.

JNK Activation Controls COX-2 Induction During MPTP-Induced Death. Stress-activated protein kinase JNK can regulate COX-2 transcription in mammalian cells (15). We therefore investigated whether MPTP-induced COX-2 up-regulation is a JNK-dependent event. After MPTP administration to mice there was a robust ventral midbrain activation of JNK, as evidenced by c-Jun phosphorylation (Fig. 4*G*) and, as shown above, a marked up-regulation of COX-2 (Fig. 4*H*). Conversely, in mice in which JNK activation was blocked by 1 mg/kg CEP-11004, MPTP caused almost no c-Jun phosphorylation and no COX-2 up-regulation (Fig. 4 *G* and *H*), thus demonstrating the critical role of the JNK/c-Jun pathway in MPTP-mediated COX-2 induction.

Administration of CEP-11004 at 1 mg/kg decreased MPTP-induced SNpc dopaminergic neuronal death, but failed to attenuate striatal dopaminergic fiber loss in *Ptgs2*$^{+/+}$ mice (Fig. 4 *C* and *E* and Table 1). The magnitude of neuroprotection against MPTP provided by the lack of COX-2 did not differ between CEP-11004-treated and untreated *Ptgs2*$^{-/-}$ mice (Fig. 4 *D* and *F* and Table 1). These data show that although both blockade of JNK and lack of COX-2 attenuate MPTP-induced SNpc dopaminergic neuronal death, the combination of the two strategies does not enhance neuroprotection.

COX-2 Ablation and Inhibition Do Not Impair MPTP Metabolism. The main determining factors of MPTP neurotoxic potency are its conversion in the brain to MPP^+ followed by MPP^+ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (16). To ascertain that resistance to the neurotoxic effects of MPTP provided by COX-2 ablation or inhibition was not because of alterations in any of these three key MPTP neurotoxic steps, we measured striatal levels of MPP^+ 90 min after the last injection of MPTP, striatal uptake of [^{3}H]MPP^+ into synaptosomes, and striatal MPP^+-induced lactate production, a reliable marker of mitochondrial inhibition (17). Striatal levels of MPP^+ were not lower in MPTP-injected *Ptgs2*$^{-/-}$ mice compared with *Ptgs2*$^{+/+}$ mice, regardless of whether or not mice received the JNK pathway inhibitor (see Table 3). Striatal levels of MPP^+ did not differ between MPTP-injected regular mice that either received or did not receive rofecoxib (see Table 3). The absence of the COX-2 gene or the presence of rofecoxib up to 32 μM did not affect MPP^+-induced lactate production (lactate in μM/100 mg of protein: *Ptgs2*$^{+/+}$ = 56.1 ± 1.9, *Ptgs2*$^{-/-}$ = 58.6 ± 5.2; regular mice/vehicle = 60.8 ± 3.8, regular mice/rofecoxib = 53.8 ± 7.3;

Table 1. Effect of COX-2 ablation and JNK pathway inhibition on MPTP toxicity

	SNpc: no. of TH-positive neurons			Striatum: TH-positive fibers, OD × 100		
Treatment	*Ptgs2*$^{+/+}$	*Ptgs2*$^{+/-}$	*Ptgs2*$^{-/-}$	*Ptgs2*$^{+/+}$	*Ptgs2*$^{+/-}$	*Ptgs2*$^{-/-}$
Saline	9,153 ± 328	9,104 ± 643	9,200 ± 643	11.9 ± 2.7	12.2 ± 1.9	11.5 ± 1.9
MPTP	5,228 ± 283*†‡	6,296 ± 356*‡	7,600 ± 610	1.9 ± 1.0*‡	2.6 ± 0.6*‡	7.3 ± 0.4
MPTP/CEP-11004	6,933 ± 501	—	8,420 ± 799	2.4 ± 0.5*‡	—	7.4 ± 0.3

Values are mean ± SEM for four to eight mice per group. *, $P < 0.05$ compared with the other groups of saline-treated mice; †, $P < 0.05$ compared with MPTP-injected *Ptgs2*$^{+/+}$ mice treated with the JNK pathway inhibitor CEP-11004; ‡, $P < 0.05$ compared with all three groups of *Ptgs2*$^{-/-}$ mice.

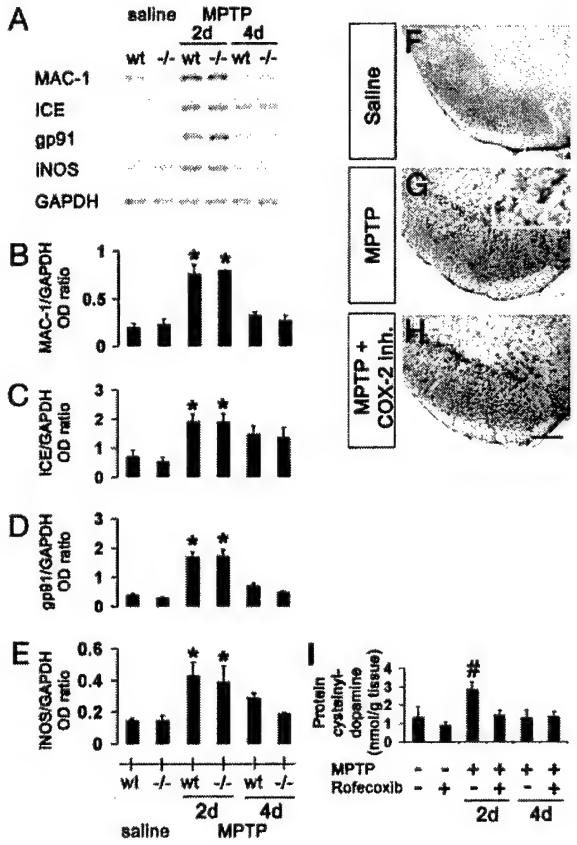

Fig. 6. Expression of inflammatory and oxidative stress markers after MPTP. Two days after MPTP injection, mRNA expression of MAC-1 (*A* and *B*), ICE (*A* and *C*), gp91 (*A* and *D*), and iNOS (*A* and *E*) are increased in the ventral midbrain and none is attenuated by COX-2 ablation. MAC-1 immunoreactivity is minimal in saline-injected mice in ventral midbrain (*F*), but is increased after MPTP injection (*G*; *Inset* shows MPTP-induced microglial activation at higher magnification). (*H*) COX-2 inhibition does not attenuate MPTP-induced microglial activation. (*I*) MPTP increases ventral midbrain protein-bound cysteinyl-dopamine, which is blocked by rofecoxib. Data are mean ± SEM for four to six mice per group. *, $P < 0.05$, compared with saline treated groups; #, $P < 0.05$, compared with the other five groups (Newman–Keuls posthoc test). (Scale bar, 250 μm.)

mean ± SEM for six mice per group). Striatal uptake of [^{3}H]MPP$^+$ was not impaired in *Ptgs2*$^{-/-}$ mice, compared with *Ptgs2*$^{+/+}$ mice, with an IC_{50} of 226.3 ± 21 nM for *Ptgs2*$^{-/-}$ mice and 195.3 ± 6.38 nM for their wild-type littermates (mean ± SEM for three mice per group). These findings suggest that COX-2-mediated neurotoxicity during MPTP-induced neuronal death operates either in parallel or downstream to MPTP's key metabolic steps.

COX-2 Modulation Does Not Alleviate MPTP-Associated Microglial Activation. Given the proinflammatory role of prostanoids such as PGE_2, we investigated the potential involvement of SNpc dopaminergic neuron production of prostaglandins in MPTP-associated microglial activation. As shown before (11, 18), there is a robust microglial activation in mice after MPTP administration. This activation was evidenced by increased contents of MAC-1, iNOS, gp91, and ICE mRNAs in ventral midbrains (Fig. 6 *A–E*), as well as by increased numbers of MAC-1-positive cells in both SNpc (Fig. 6*G*) and striatum (data not shown). Whereas both COX-2 abrogation and inhibition attenuated MPTP-mediated death, neither prevented the microglial response described above (RT-PCR: Fig. 6 *A–E*, data not shown for COX-2 inhibition; immunostaining for MAC-1: Fig. 6 *F–H*; data not shown for COX-2 ablation). Thus, COX-2 plays a negligible role in the microglial activation and the production of microglial-derived noxious factors after MPTP intoxication.

COX-2 Mediates Oxidative Stress During MPTP-Induced Neurodegeneration. Aside from production of extracellular prostanoids, COX-2 can also damage intracellular protein-bound sulfhydryl groups through the oxidation of catechols such as dopamine (19). To investigate whether such a mechanism is in play here, we quantified ventral midbrain content of protein 5-cysteinyl-dopamine, a stable modification engendered by the COX-related oxidation of dopamine (19). In saline-injected mice, baseline levels of protein 5-cysteinyl-dopamine were slightly lower in mice treated than those not treated with rofecoxib (Fig. 6*I*). In MPTP-injected mice that did not receive rofecoxib, protein 5-cysteinyl-dopamine levels were >2-fold higher than in their saline-injected counterparts (Fig. 6*I*). In contrast, in MPTP-injected mice that did receive rofecoxib, there was no significant increase in protein 5-cysteinyl-dopamine levels compared with their saline controls (Fig. 6*I*).

Discussion

This study shows an up-regulation of COX-2 in the brain regions that house nigrostriatal dopaminergic neurons in both MPTP mice and human PD samples. Increased COX-2 expression was associated with increased PGE_2 tissue content, thus indicating that the increased COX-2 is catalytically active. However, we found that ventral midbrain PGE_2 reflects mainly COX-1 activity in both normal and MPTP-injured mice. Although affected brain regions in MPTP and PD are cellularly heterogeneous, conspicuous COX-2 immunoreactivity was essentially found in SNpc dopaminergic neurons from MPTP-treated mice and postmortem PD samples. This finding raises the possibility that COX-2 up-regulation could amplify the neurodegenerative process specifically in SNpc dopaminergic neurons, thus rendering these neurons more prone than any other neurons to succumb to MPTP toxicity or PD injury.

Consistent with the involvement of COX-2 in MPTP and PD neurodegenerative processes, approximately twice as many SNpc dopaminergic neurons and striatal dopamine fibers survived in *Ptgs2*$^{-/-}$ mice compared with their wild-type littermates after MPTP administration. These results agree with the previous demonstrations that COX-2 modulation mitigates MPTP-mediated SNpc dopaminergic neurotoxicity in mice (20, 21). Because COX-2 can also exert deleterious effects unrelated to its catalytic activity (14), it must be noted that lack of COX-2 protein and inhibition of COX-2 by rofecoxib produced comparable protection of SNpc dopaminergic neurons against MPTP; striatal dopaminergic fibers were better protected by COX-2 ablation than by inhibition. It can thus be concluded that the deleterious effect of COX-2, at least on SNpc dopamine neurons in the MPTP model, and probably in PD, relies on COX-2 catalytic activity. Unlike ablation of COX-2, ablation of COX-1 failed to produce any protection against MPTP, thus indicating that induction of COX-2 expression, but not COX-1 or COX-1 gene products (e.g., COX-3; ref. 22), is instrumental in MPTP neurotoxicity.

Our data confirm the activation of the JNK/c-Jun signaling pathway after MPTP administration (23) and demonstrate that the blockade of this pathway by CEP-11004 at a concentration that inhibits c-Jun phosphorylation also inhibits COX-2 induction. In mice lacking both JNK-2 and JNK-3 genes, we found that MPTP fails to cause any phosphorylation of c-Jun or induction of COX-2 (S.H., M.V., P.T., R.J. Davis, S.P., E.C. Hirsch, P. Rakic, and R. A. Flavell, unpublished data). These results support a critical role for the JNK/c-Jun signaling pathway in the regulation of COX-2 expression in SNpc dopaminergic neurons after MPTP administration. However, COX-2 ablation attenuated MPTP-induced SNpc dopaminergic neuronal and striatal dopaminergic fiber loss, whereas JNK pathway inhibition protected only against SNpc neuronal death. This finding suggests that, in the absence of any COX-2 induction, residual COX-2 proteins in CEP-11004-treated

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mice suffice to damage at least striatal dopaminergic fibers, which are more sensitive to MPTP than to SNpc dopaminergic neurons. We also show that the combination of JNK blockade and COX-2 ablation did not confer neuroprotection against MPTP beyond that produced by COX-2 ablation alone. It can thus be concluded that among the host of genes regulated by JNK, COX-2 may be the mediator of JNK's deleterious effects on SNpc dopaminergic neurons in the MPTP model of PD.

COX-2 toxicity is presumably mediated by its production of inflammatory prostanoids. Accordingly, neurons expressing COX-2 would cause their own demise through a harmful interplay with glial cells: COX-2-positive neurons release PGE_2, which promotes the production of microglial-derived mediators, which, in turn, help in killing neurons. Although we have previously demonstrated that activated microglia and derived factors do amplify MPTP-induced neurodegeneration (11), the present study shows that COX-2 modulation alters neither the morphological nor the functional correlates of microglial activation after MPTP administration. Therefore, neuronal COX-2 cytotoxicity in this model of PD does not appear to be linked to the inflammatory response. This view is consistent with our finding that most of the ventral midbrain PGE_2 originates not from COX-2, but from COX-1.

Alternatively, neuronal COX-2 overexpression may kill neurons in a cell-autonomous manner (5, 6, 24). Relevant to the leading pathogenic hypothesis for PD (25) is the fact that COX-2 cell-autonomous toxicity may arise from the formation of reactive oxygen species generated during COX peroxidase catalysis of PGG_2 conversion to PGH_2 (26). On donation of electrons to COX, cosubstrates such as dopamine become oxidized to dopamine-quinone (19), which is highly reactive with glutathione and protein amino acids such as cysteine, tyrosine, and lysine. Supporting the occurrence of such an oxidative process after MPTP injection is the marked increase in ventral midbrain protein cysteinyl-dopamine content, a fingerprint of protein cysteinyl attack by dopamine-quinone (19), in MPTP-intoxicated mice. We also demonstrated the COX-2 dependency of this toxic event by showing that COX-2 inhibition prevented the rise in protein cysteinyldopamine seen after MPTP injections. The deleterious consequences of dopamine-quinone can include depletion of vital antioxidants such as glutathione, inactivation of critical enzymes such as TH (27), and accumulation of α-synuclein protofibrils, a proposed key event in PD pathogenesis (28). Given these findings, it is thus undeniable that COX-2 up-regulation in SNpc dopaminergic neurons can unleash an array of oxidative assaults, which ultimately may play a decisive role in determining the fate of these neurons in the MPTP model and in PD itself.

Collectively, our data provide evidence for COX-2 up-regulation in MPTP and PD and support a significant role for COX-2 in both the mechanism and the specificity of MPTP- and PD-induced SNpc dopaminergic neuronal death. The present study suggests that inhibition of COX-2 may be a valuable target for the development of new therapies for PD aimed at slowing the progression of the neurodegenerative process.

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NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease

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Parkinson's disease (PD) is a neurodegenerative disorder of uncertain pathogenesis characterized by a loss of substantia nigra pars compacta (SNpc) dopaminergic (DA) neurons, and can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both inflammatory processes and oxidative stress may contribute to MPTP- and PD-related neurodegeneration. However, whether inflammation may cause oxidative damage in MPTP and PD is unknown. Here we show that NADPH-oxidase, the main reactive oxygen species (ROS)-producing enzyme during inflammation, is up-regulated in SNpc of human PD and MPTP mice. These changes coincide with the local production of ROS, microglial activation, and DA neuronal loss seen after MPTP injections. Mutant mice defective in NADPH-oxidase exhibit less SNpc DA neuronal loss and protein oxidation than their WT littermates after MPTP injections. We show that extracellular ROS are a main determinant in inflammation-mediated DA neurotoxicity in the MPTP model of PD. This study supports a critical role for NADPH-oxidase in the pathogenesis of PD and suggests that targeting this enzyme or enhancing extracellular antioxidants may provide novel therapies for PD.

Parkinson's disease (PD) is a common neurodegenerative disease characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (1). Its main neuropathological feature is the loss of the nigrostriatal dopamine (DA)-containing neurons, whose cell bodies are in the substantia nigra pars compacta (SNpc) and nerve terminals are in the striatum (2). Except for a handful of inherited cases related to known gene defects, PD is a sporadic condition of unknown pathogenesis (1).

Epidemiological studies suggest that inflammation increases the risk of developing PD (3). Consistent with this view, experimental models of PD show that inflammatory factors may trigger or modulate SNpc DA neuronal death (4–6). Among inflammatory mediators capable of promoting neurodegeneration are microglial-derived reactive oxygen species (ROS). These may deserve particular attention, because oxidative stress is a leading pathogenic hypothesis of PD (7).

A significant source of ROS during inflammation is NADPH-oxidase, which is a multimeric enzyme composed of $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits (8). In resting microglia, NADPH-oxidase is inactive because $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, which are present in the cytosol as a complex, are separated from $gp91^{phox}$ and $p22^{phox}$, which are transmembrane proteins. Upon microglial activation, $p47^{phox}$ becomes phosphorylated and the entire cytosolic complex translocates to the membrane, where it assembles with $gp91^{phox}$ and $p22^{phox}$, thus forming a NADPH-oxidase entity now capable of reducing oxygen to superoxide radical (O_2^-), which in turn gives rise to the production of other secondary reactive oxidants (8).

Although NADPH-oxidase is critical to the killing of invading microorganisms in infections through its abundant and sustained production of O_2^- (8), its role in noninfectious chronic neurodegenerative processes, such as PD, is not known. In the present study, we show not only that the NADPH-oxidase main subunit $gp91^{phox}$ is up-regulated in the SNpc of PD and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice, but also that NADPH-oxidase inactivation attenuates MPTP neurotoxicity by mitigating inflammation-mediated oxidative attack on SNpc neurons. These findings indicate that NADPH-oxidase-induced oxidative stress is instrumental in SNpc DA neurodegeneration caused by MPTP, and suggest that NADPH-oxidase is a valuable therapeutic target for the development of neuroprotective strategies for PD.

Materials and Methods

Animals and Treatment. Eight-week-old male C57BL/6 mice (Charles River Breeding Laboratories), $gp91^{phox}$-deficient mice (B6.129S6-$Cybb^{tm1din}$, The Jackson Laboratory), and their WT littermates were used. Mice received four i.p. injections of MPTP-HCl (16 mg/kg free base; Sigma) dissolved in saline at 2-h intervals, and were killed 0–14 days after the last injection. Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (9). Minocycline (2 × 45 mg/kg per day; Sigma) was given to MPTP mice as described (5). Bovine erythrocyte superoxide dismutase 1 (SOD1; 20 units/h; Sigma) was infused into the left striatum with an osmotic minipump (Alzet, Palo Alto, CA) starting 1 day before and stopping 6 days after the MPTP injections. This protocol was in accordance with the National Institutes of Health guidelines for use of live animals and was approved by the Institutional Animal Care and Use Committee of Columbia University (New York). Striatal 1-methyl-4-phenylpyridinium levels were determined by HPLC as described (5).

RNA Extraction and RT-PCR. Total RNA was extracted as described (5). The primer mouse sequences were as follows: $gp91^{phox}$, 5′-CAGGAGTTCCAAGATGCCTG-3′ (forward) and 5′-GATTGGCCTGAGATTCATCC-3′ (reverse); $p67^{phox}$, 5′-CAGCCAGCTTCGGAACATG-3′ (forward) and 5′-GACAGTACCAGGATTACATC-3′ (reverse); macrophage antigen complex 1 (Mac-1), 5′-TTCTCATGGTCACCTCCTGC-3′ (forward) and 5′-GGTCTGACCATCTGAACCTG-3′ (reverse); GAPDH, 5′-GTTTCTTACTCCTTGGAGGCCAT-3′ (forward) and 5′-TGATGACATCAAGAAGTGGTGAA-3′ (reverse). PCR was carried out for 29 cycles for $gp91^{phox}$, 27 cycles for $p67^{phox}$ and Mac-1, and 20 cycles for GAPDH. Each cycle

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Abbreviations: Mac-1, macrophage antigen complex 1; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; DA, dopamine/dopaminergic; SOD1, superoxide dismutase 1.

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consisted of 30 s of denaturation at 94°C, 40 s of annealing at 55°C, and 60 s of extension at 72°C, followed by a final 7-min extension at 72°C. Products were quantified by a phosphor imager (Bio-Rad).

Digoxigenin-Labeled cRNA Probe and *in Situ* Hybridization. Antisense and sense RNA probes were prepared by *in vitro* transcription from a mouse gp91^{phox} cDNA fragment (nucleotides 1020–1493; GenBank accession no. U43384) by using SP6/T7 RNA polymerase in the presence of digoxigenin-linked UTP (Roche Molecular Biochemicals) according to the supplier's instructions. Frozen midbrain sections (14 μm thick) were incubated with the antisense or sense (for control) digoxigenin-labeled probes. Hybridization signal was detected by 5-bromo-4-chloro-indolyl-phosphatase and nitroblue tetrazolium.

Immunohistochemistry and Quantitative Morphology. Mouse brains were fixed and processed for immunostaining as described (5). Primary Abs were as follows: for mouse sections, monoclonal anti-mouse gp91^{phox} (1:1,000; Transduction Laboratories, Lexington, KY), rat anti-MAC-1 (1:200; Serotec), monoclonal anti-tyrosine hydroxylase (TH; 1:1,000; Chemicon), and polyclonal anti-TH (1:1,000; Calbiochem, San Diego); for human sections, monoclonal anti-human gp91^{phox} (gift from Genentech) and monoclonal anti-human CD68 (DAKO). Immunolabeling was visualized by using 3,3′-diaminobenzidine (brown), VECTOR SG (blue/gray), 3-amino-9-ethylcarbazole (red), or fluorescein and Texas red (all from Vector Laboratories).

Total numbers of TH-positive SNpc neurons were counted by stereology by using the optical fractionator method described previously (6). Striatal density of TH immunoreactivity was determined as described (5).

Western Blots. Total, cytosolic, and plasma membrane proteins were prepared as described (5). Primary Abs were as follows: for mouse proteins, monoclonal anti-mouse p67^{phox} (1:1,000; Transduction Laboratories), polyclonal anti-gp91^{phox} (1:5,000, gift from M. C. Dinauer, Indiana University, Indianapolis), and polyclonal anti-calnexin (1:5,000; Stressgen Biotechnologies, Victoria, Canada); for human proteins, monoclonal anti-human gp91^{phox} (1:500, Genentech). A monoclonal anti-β-actin (1:5,000; Sigma) was used for both mouse and human proteins. Bound primary Ab was detected by using a horseradish peroxidase-conjugated secondary Ab against IgG and a chemiluminescent substrate (SuperSignal Ultra, Pierce). Films were quantified by using the NIH IMAGE analysis system.

***In Situ* Visualization of O_2^- and O_2^--Derived Oxidant Production.** *In situ* visualization of O_2^- and O_2^--derived oxidant production was assessed by hydroethidine histochemistry (10). At selected time points after MPTP administration, mice were injected i.p. with 200 μl of PBS containing 1 μg/μl hydroethidine (Molecular Probes) and 1% DMSO. Brains were harvested 15 min later and frozen on dry ice. Midbrain sections (14 μm thick) mounted onto gelatin-coated glass slides were examined for hydroethidine oxidation product, ethidium accumulation, by fluorescence microscopy (excitation, 510 nm; emission, 580 nm). The same tissue sections were used for Mac-1 immunohistochemistry.

Protein carbonyls were detected after derivatization of brain homogenates with 2,4-dinitrophenylhydrazine by using a modification of the method described by Levine *et al.* (11). The concentration of protein carbonyls was calculated from the difference in absorbance at 360 nm between the underivatized and 2,4-dinitrophenylhydrazine-derivatized samples normalized to the protein concentration. The extinction coefficient of 21 $mM^{-1}{\cdot}cm^{-1}$ was applied to calculate the concentration of protein carbonyls.

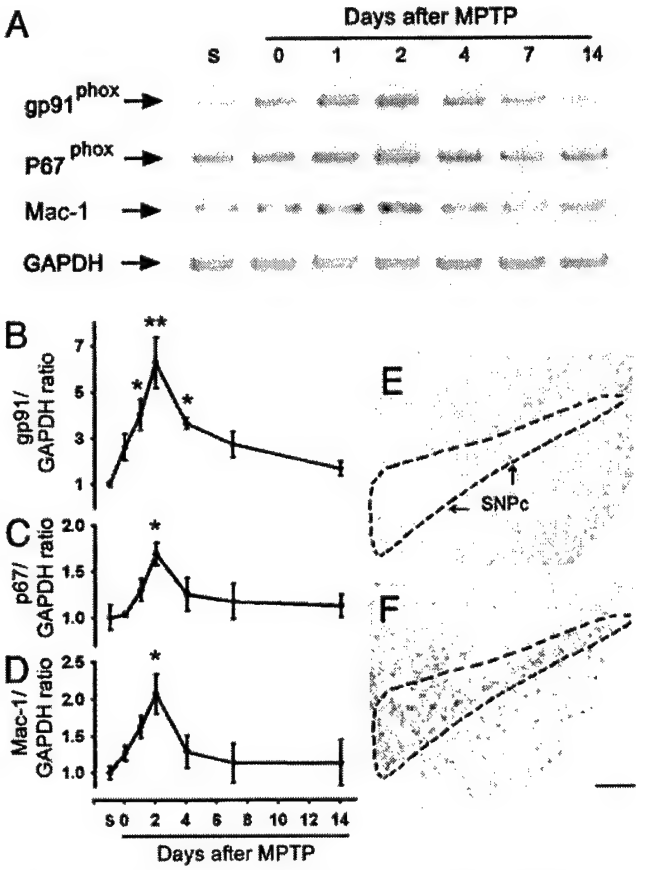

Fig. 1. (*A–D*) RT-PCR shows ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 mRNA levels in saline-injected (S) and MPTP-injected mice from 0 to 14 days after injections. SNpc gp91^{phox} mRNA labeling is negligible in saline-injected mice (*E*), whereas it is copious in MPTP-injected mice at 2 days (*F*). *, $P < 0.05$; **, $P < 0.001$, more than saline-treated mice ($n = 4–6$ per time point). (Scale, 2.5 mm.)

Human Samples. Age at death and interval from death to tissue processing (mean ± SEM) were as follows: for the control group ($n = 3$), 72.2 ± 8.8 y and 13.0 ± 3.5 h, respectively; for the PD group ($n = 6$), 77.2 ± 2.3 y and 10.1 ± 2.4 h, respectively. For the PD patients, the mean duration of disease was 16.8 ± 2.3 y.

Statistical Analysis. Values represent means ± SEM. Differences among means were analyzed by using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were analyzed by Newman–Keuls post hoc testing. The null hypothesis was consistently rejected at the 0.05 level.

Results

NADPH-Oxidase Is Induced in Mouse Ventral Midbrain During MPTP Neurotoxicity. To define the temporal relationship between NADPH-oxidase expression and MPTP neurotoxicity, contents of ventral midbrain (brain region containing SNpc) membrane-bound subunit gp91^{phox} and cytosolic subunit p67^{phox} mRNA were assessed throughout the time course of MPTP-induced SNpc DA neurodegeneration (12). In saline-injected mice, ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 (microglial marker) mRNAs were low (Fig. 1 *A–D*). In contrast, in MPTP-injected mice, ventral midbrain gp91^{phox}, p67^{phox}, and Mac-1 mRNAs

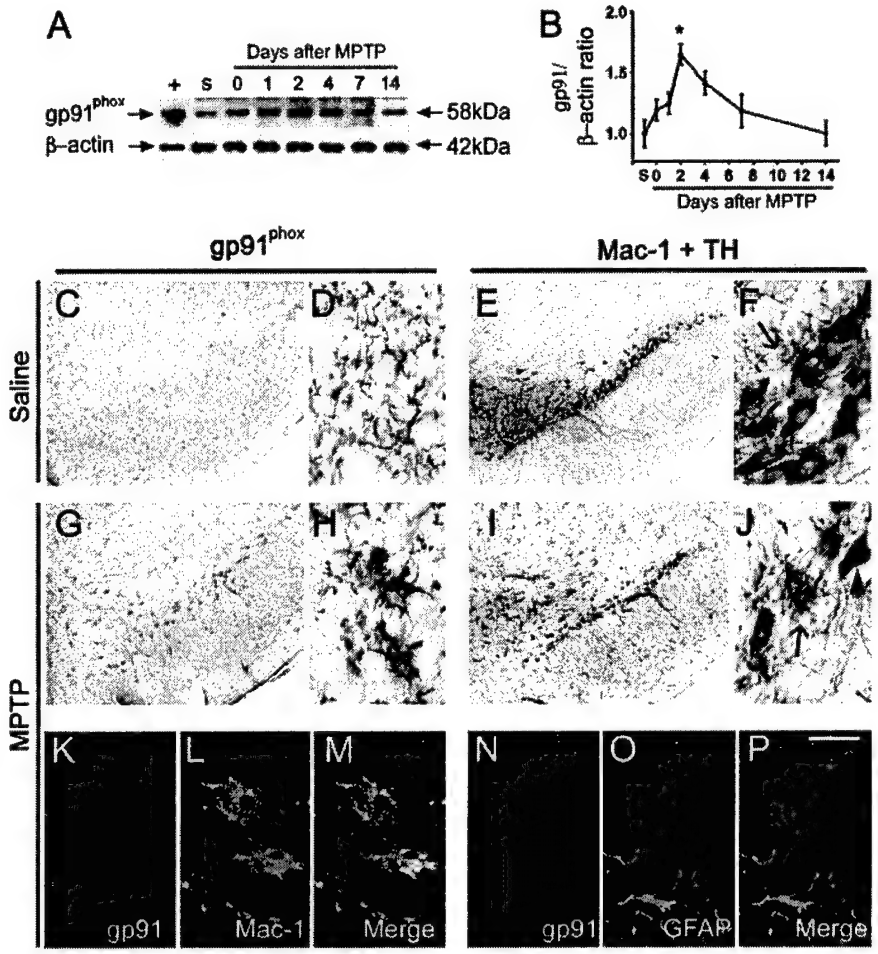

Fig. 2. Western blot shows the time-dependent induction of gp91^{phox} in mouse ventral midbrain after MPTP injections. +, Mouse macrophage lysate; s, saline. In saline-injected mice, gp91^{phox} immunoreactivity (*C* and *D*, brown) is mild and localized in resting microglia, which are not abundant in the SNpc, as shown (*E* and *F*) by Mac-1 labeling (brown, arrow) and are intermingled with TH-positive neurons (gray-blue). Two days after MPTP injections, numerous gp91^{phox}-positive cells are seen in the SNpc (*G* and *H*). These cells resemble activated microglial cells (*H* vs. *J*, arrow). At this point there are many fewer TH-positive neurons (*I* and *J*, arrowhead). Confocal microscopy shows that all gp91^{phox}-positive cells are Mac-1-positive, thus confirming their microglial origin (*K* and *L*). Conversely, no gp91^{phox}-positive cells are glial fibrillary acidic protein-positive cells, thus excluding their astrocytic origin (*N–P*). *, $P < 0.05$, more than saline-treated mice (n = 6 per time point). [Scale bar, 2.5 mm (*C*, *E*, *G*, and *I*); 0.25 mm (*D*, *F*, *H*, and *J*); and 0.2 mm (*K–P*).]

increased in a time-dependent manner after MPTP injections (Fig. 1 *A–D*).

In saline-injected mice, nonradioactive *in situ* hybridization for gp91^{phox}, which is one of the main functional subunits of NADPH-oxidase, revealed no specific labeling in ventral midbrain (Fig. 1*E*), whereas in MPTP-injected mice there was conspicuous specific labeling over the SNpc at 2 days after MPTP injections (Fig. 1*F*). Thus, these results indicate that NADPH-oxidase is induced after MPTP administration specifically in the area where the demise of DA neurons arises in this model of PD.

NADPH-Oxidase Is Expressed in Activated Microglia After MPTP Injection. Consistent with the mRNA data, ventral midbrain gp91^{phox} protein contents rose in a time-dependent manner after MPTP injections (Fig. 2 *A* and *B*). In cell cultures, NADPH-oxidase has been identified in different cell types, including neurons (13). In saline-injected mice, mild gp91^{phox} immunoreactivity was seen throughout the substantia nigra (Fig. 2*C*) without greater gp91^{phox} immunolabeling in the SNpc, which hosts the TH-positive neurons (Fig. 2 *E* and *F*). Immunoreactivity of gp91^{phox} was in small cells with thin ramifications (Fig. 2*D*) reminiscent of resting microglia (Fig. 2*F*). In MPTP-injected mice, robust gp91^{phox} immunoreactivity was seen specifically in the SNpc (Fig. 2*G*) in larger cells with thick, shorter ramifications (Fig. 2*H*) reminiscent of activated microglia (Fig. 2*J*). Similar immunohistochemical gp91^{phox} alterations were seen in the striatum, which contains the nerve terminals of the projecting SNpc DA neurons, between the saline- and MPTP-injected mice (data not shown). By confocal microscopy, gp91^{phox} immunoreactivity appeared to colocalize with Mac-1 (Fig. 2 *K–M*). Conversely, gp91^{phox} immunoreactivity did not colocalize either with the astrocytic marker glial fibrillary acidic protein (Fig. 2 *N–P*) or with TH (data not shown). Thus, these results demonstrate that, after MPTP administration, SNpc microglia become activated at the site of NADPH-oxidase induction.

Expression of gp91^{phox} Is Increased in PD Midbrain. Consistent with the finding in the MPTP mice, postmortem SNpc samples from sporadic PD patients had higher gp91^{phox} protein contents than controls (Fig. 3 *A* and *B*). In these autopsy specimens, cellular gp91^{phox} immunoreactivity was barely identified in controls (Fig. 3*C*) but was strong in PD midbrain sections, where it was identified in microglial cells (Fig. 3*D*). The similarity of the

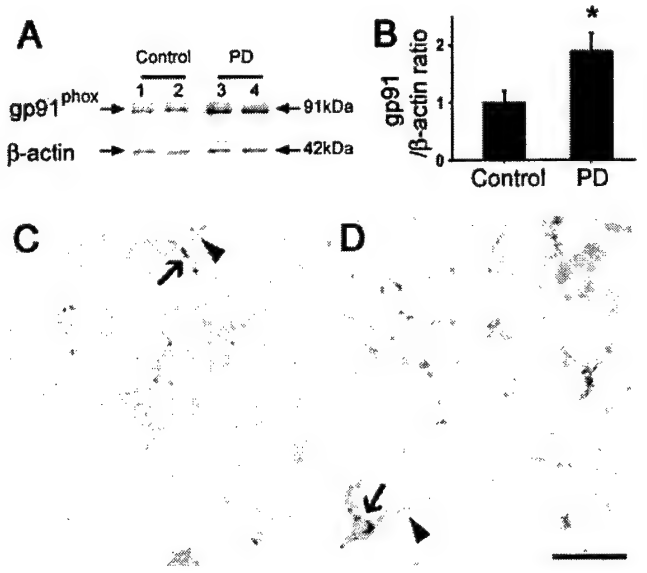

Fig. 3. (*A*) Representative Western blots illustrating the increase in ventral midbrain gp91^{phox} protein content in two PD and two controls. (*B*) Bar graph showing mean Western blot gp91^{phox}/β-actin ratios ± SEM for six PD and three control ventral midbrain samples. (*C* and *D*) Representative gp91^{phox} immunostaining that shows positive cells in PD samples (arrowhead, gray-blue, membrane labeling) colocalizing with the microglial marker CD68 (arrow, red, cytosol labeling), but not with neuromelanin (brown pigment). *, $P < 0.05$, higher than controls. (Scale bar, 0.5 mm.)

gp91^{phox} alterations between the MPTP mice and the PD postmortem specimens validates the use of the MPTP experimental model to study the role of NADPH-oxidase in the PD neurodegenerative process.

The Lack of gp91^{phox} Abates MPTP-Associated ROS Production. In saline-injected mice, ventral midbrain O_2^- and O_2^--derived oxidant production, evidenced by ethidium fluorescence, was minimal (Fig. 4*A*). In contrast, in MPTP-treated mice, ventral midbrain production of O_2^- or O_2^--derived oxidants shown by ethidium fluorescence was increased by 12 h (data not shown), was maximal by 2 days (Fig. 4*C*), and remained elevated at 7 days after MPTP (data not shown). SNpc ethidium fluorescence coincided with the location and the time course of microglial activation seen after MPTP administration (Fig. 4 *C* and *D*).

In mutant mice lacking the gp91^{phox} subunit, no translocation of the cytosolic p67^{phox} subunit to the plasma membrane was seen after MPTP injections (Fig. 4 *I* and *J*), which is mandatory for NADPH-oxidase to become catalytically competent (8). Unlike WT littermates (Fig. 4 *C* and *D*), mutant mice with defective NADPH-oxidase failed to show any increase in SNpc ethidium fluorescence (Fig. 4*E*), despite normal microglial activation after MPTP administration (Fig. 4*F*). WT mice treated with minocycline (i.e., antibiotic that blocks microglial activation) showed no increase in SNpc ethidium fluorescence (Fig. 4*G*) and no microglial activation after MPTP administration (Fig. 4*E*). Thus, these findings demonstrate that during the MPTP neurotoxic process there is an increased production of ROS in the SNpc that originates from activated microglial cells and is mediated by NADPH-oxidase.

NADPH-Oxidase Defect Protects Against MPTP Neurodegeneration. In the ventral midbrain of saline-injected mice, the stereological counts of SNpc TH-positive neurons did not differ between gp91^{phox}-deficient mice and their WT littermates (Fig. 5*A*). In MPTP-injected mice, the numbers of SNpc TH-positive neurons

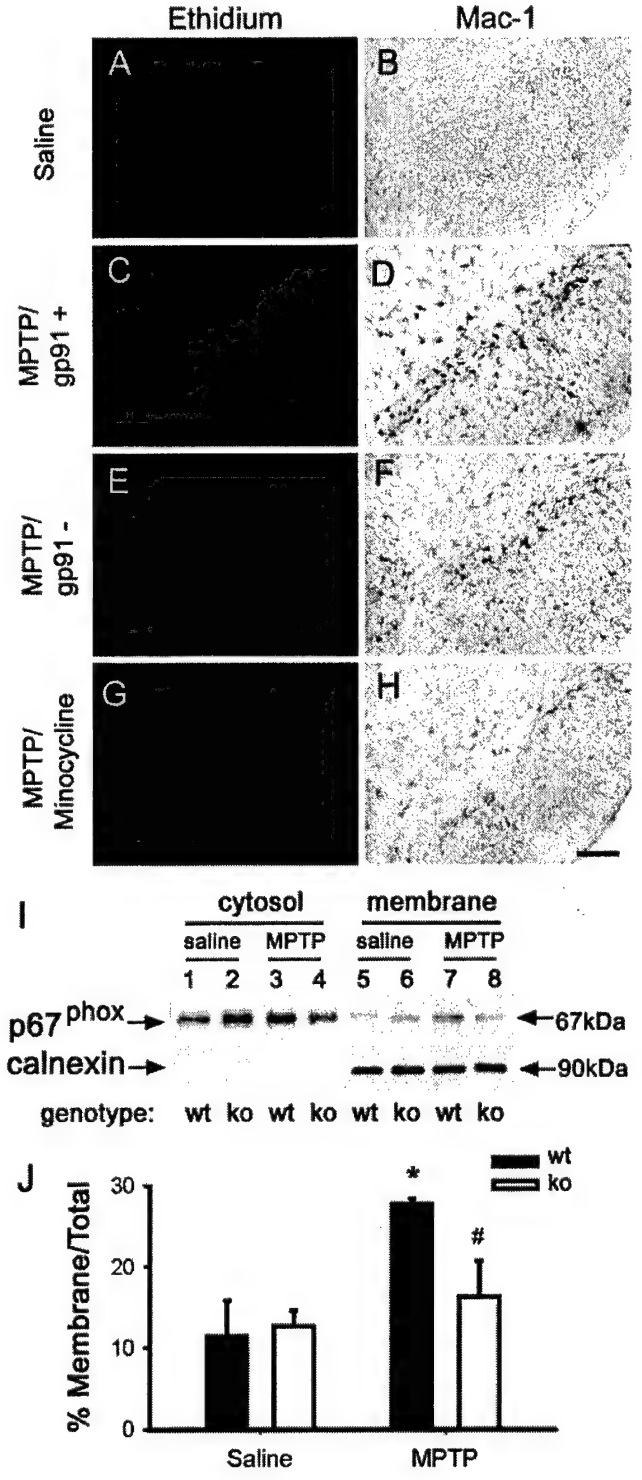

Fig. 4. Ethidium fluorescence (*A*) and Mac-1 immunostaining (*B*) are minimal in the saline-treated mice. By 2 days after MPTP injections, SNpc ethidium fluorescence is increased in WT mice (*C*) and is absent in gp91^{phox}-deficient mice (*E*) and minocycline-treated WT mice (*G*). Microglial activation is prevented by minocycline (*H*) but is normal in gp91^{phox}-deficient mice (*E*). MPTP stimulates NADPH-oxidase activation, as evidenced by p67^{phox} translocation from the cytosol to the plasma membrane in WT mice (wt), but not in gp91^{phox}-deficient mice (ko) (*I* and *J*); the membrane protein calnexin is used to normalize the data. Data are means ± SEM for four to six samples per group. *, $P < 0.05$, higher than controls; #, $P < 0.05$, less than MPTP-injected WT mice, but not different from both saline-injected groups.

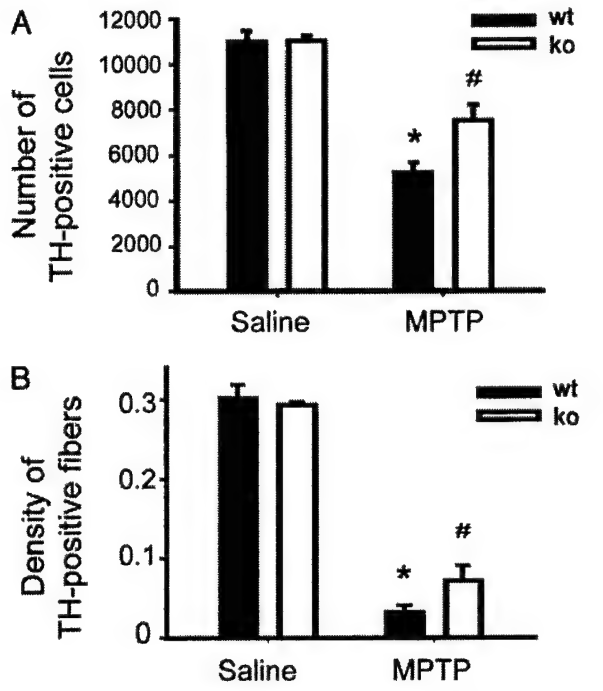

Fig. 5. Stereological counts of TH-positive neurons in the SNpc (*A*) and optical density of striatal DA fibers (*B*) are higher in gp91^{phox}-deficient mice (ko) compared with their WT littermates (wt) 7 days after MPTP injections (n = 4–8 samples per group). *, $P < 0.05$, less than saline-injected mice; #, $P < 0.05$, higher than MPTP-injected WT mice.

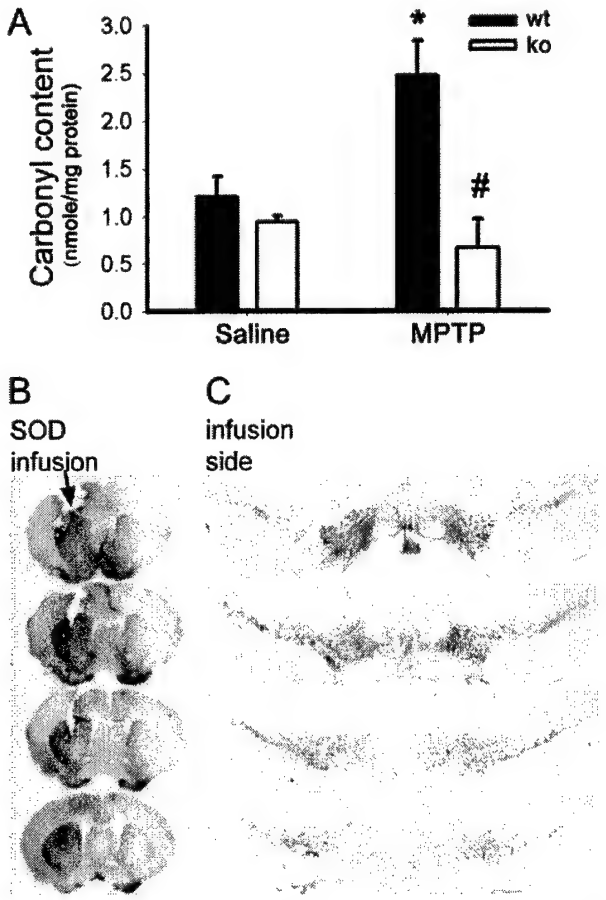

Fig. 6. (*A*) Ventral midbrain carbonyl content, used as a marker of protein oxidative damage, is increased at 2 days after MPTP injections in WT mice (wt), but not in gp91^{phox}-deficient mice (ko). Infusion of SOD1 into the left striatum attenuates the striatal (*B*) and the SNpc lesion on the infused side, but not on the contralateral, noninfused side (*C*) after a systemic injection of MPTP. *, $P <$ 0.05, higher than controls; #, $P < 0.05$, less than MPTP-injected WT mice, but not different from the two saline-injected groups.

were reduced in the two groups of animals (Fig. 5*A*). However, the loss was smaller in gp91^{phox}-deficient mice compared with their WT counterparts (Fig. 5*A*). In the striatum of saline-injected mice, the density of TH-positive nerve fibers was similar between gp91^{phox}-deficient mice and their WT littermates (Fig. 5*B*). Like for the number of SNpc TH-positive neurons, in MPTP-injected mice the density of striatal TH-positive nerve fibers was less reduced in the gp91^{phox}-deficient mice than in their WT counterparts (Fig. 5*A*). Because MPTP neurotoxic potency on the nigrostriatal pathway correlates linearly with 1-methyl-4-phenylpyridinium levels in the striatum (14), the content of this active metabolite of MPTP between the two genotypes was evaluated. There were no differences in striatal levels of 1-methyl-4-phenylpyridinium between the gp91^{phox}-deficient mice (17.8 ± 1.4 μg/g striatum; $n = 5$) and WT littermates (17.7 ± 2.0 μg/g striatum; $n = 5$; $P > 0.05$). These results show that NADPH-oxidase participates in the MPTP neurotoxic process affecting DA cell bodies in the SNpc and nerve fibers in the striatum by a mechanism unrelated to an alteration in MPTP toxicokinetics.

NADPH-Oxidase Damages Ventral Midbrain Proteins. To assess the extent of NADPH-oxidase-related oxidative damage, protein carbonyl levels were determined in ventral midbrain of gp91^{phox}-deficient and WT mice after saline or MPTP administration. In saline-injected mice, the levels of ventral midbrain protein carbonyls were similar between the two groups of animals (Fig. 6*A*). In MPTP-injected WT mice, levels of ventral midbrain protein carbonyls were increased (Fig. 6*A*), but in gp91^{phox}-deficient mice they were not different from controls (Fig. 6*A*).

MPTP-Induced Neurotoxicity Is Attenuated by Scavenging Extracellular Superoxide. To test the noxious role of extracellular ROS, the membrane-impermeant enzyme SOD1 was infused into the left striatum. In the MPTP-injected mice, there was a protection of striatal TH-positive fibers on the infused side compared with the noninfused side (Fig. 6*B*). There was also a preservation of SNpc TH-positive cell bodies ipsilateral to the infused side compared with the contralateral noninfused side (Fig. 6*C*). These findings demonstrate the importance of the oxidative stress that emanates from the extracellular space on the demise of neighboring DA neurons.

Discussion

This study shows that the microglial activation in MPTP and PD SNpc specimens is associated with an induction of NADPH-oxidase. This up-regulation correlates topographically and temporally with the DA neurodegenerative changes seen in MPTP mouse and human PD brains. It also parallels the production of ROS seen in the SNpc by 2 days after MPTP injections. The use of minocycline and mutant mice deficient in gp91^{phox} demonstrates collectively that ROS production originates from activated microglia and, within these cells, from NADPH-oxidase.

In the MPTP model, ROS can emanate from both cytosol and mitochondria of DA neurons (15–18). Rise of markers reflecting oxidative damage in the nigrostriatal DA pathway culminates during the first 24 h after MPTP injections (19, 20). In contrast, SNpc NADPH-oxidase-mediated ROS attack becomes signifi-

cant by 2 days after MPTP injections. Therefore, nigrostriatal DA neurons may be subjected first to an intracellular oxidative insult, and then to an extracellular oxidative insult mediated by activated microglia.

Mutant mice deficient in gp91^{phox} exhibited less ventral midbrain protein carbonyl contents and more SNpc DA neurons than their WT littermates after MPTP injections. These results prove that NADPH-oxidase is instrumental in the MPTP neurotoxic process. Activated microglia can also exert deleterious effects unrelated to ROS. Relevant to this notion, mutant mice deficient in gp91^{phox}, despite being defective in NADPH-oxidase, showed no evidence of impaired activation of microglial cells in response to MPTP. Lack of gp91^{phox} expression was also not associated with alteration in the formation of 1-methyl-4-phenylpyridinium, which is the most significant modulator of MPTP potency (14). Therefore, the resistance of gp91^{phox}-deficient mice to MPTP results from the defect of NADPH-oxidase and the consequent reduction of O_2^- formation, and not from either an impaired microglial effector function or an altered MPTP metabolism.

Activated NADPH-oxidase produces O_2^- inward into intracellular vesicles and outward into the extracellular space (8). Neurons located in the vicinity of activated microglial cells may thus have their plasma membrane proteins and lipids exposed to NADPH-oxidase-derived O_2^- and other secondary oxidants, such as hydrogen peroxide. Infusion of SOD1 in the striatum attenuates MPTP-induced loss of striatal DA fibers and SNpc DA neurons; the latter effect may result from a reduction of MPTP-mediated retrograde degeneration (21). This finding indicates that extracellular hydrogen peroxide may not play a great neurotoxic role in the MPTP model, because its formation should have been greatly increased by the combination of infused SOD1 and increased steady-state levels of O_2^- (22) derived from activated NADPH-oxidase. Instead, an increase in the steady-state levels of extracellular O_2^- appears to be pivotal to the killing of SNpc DA neurons.

Among the main isoforms that catalyze NO synthesis, inducible NO synthase is the most closely linked to inflammation. In keeping with this, inducible NO synthase is up-regulated in activated microglial cells both in PD and in the MPTP model (6, 23, 24). In mutant mice deficient in inducible NO synthase, MPTP causes less death of SNpc DA neurons and smaller increases in ventral midbrain nitrotyrosine levels compared with their WT counterparts (6, 24). These findings suggest that a critical part of activated microglial cytotoxicity in the MPTP model and perhaps in PD is also fulfilled by inducible NO synthase-derived NO. Given this, extracellular O_2^- toxicity in the MPTP model could derive from peroxynitrite that is formed by the diffusion-limited reaction of O_2^- with NO (25). Consistent with peroxynitrite involvement in MPTP and PD neurodegenerative processes are the demonstrations that ventral midbrain nitrotyrosine levels are increased after MPTP injections (26, 27), with overexpression of SOD1 preventing the nitration of several important proteins, such as TH (19). α-Synuclein, a presynaptic protein with critical relevance to PD etiopathogenesis, is also nitrated both in the MPTP model and in PD (28, 29).

Activated microglial cells, by generating an extracellular oxidative stress, would likely injure all cells and not solely DA neurons. One way to reconcile the anticipated nonselectivity of the injury with the selectivity of the lesions is to consider that SNpc DA neurons may be particularly vulnerable to extracellular ROS attack compared with the other cells. It is also possible that in the MPTP model and in PD, the magnitude of microglial activation and resulting oxidative stress is mild and only inflicts sublethal lesions. This would succeed in killing only neurons already compromised, as DA neurons probably are in PD and after MPTP injections.

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D-β-Hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease

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Parkinson disease (PD) is a neurodegenerative disorder characterized by a loss of the nigrostriatal dopaminergic neurons accompanied by a deficit in mitochondrial respiration. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes dopaminergic neurodegeneration and a mitochondrial deficit reminiscent of PD. Here we show that the infusion of the ketone body D-β-hydroxybutyrate (DβHB) in mice confers partial protection against dopaminergic neurodegeneration and motor deficits induced by MPTP. These effects appear to be mediated by a complex II–dependent mechanism that leads to improved mitochondrial respiration and ATP production. Because of the safety record of ketone bodies in the treatment of epilepsy and their ability to penetrate the blood-brain barrier, DβHB may be a novel neuroprotective therapy for PD.

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Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease (1). PD is clinically characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (2), and its main neuropathological feature is the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons (3).

While PD is a sporadic condition of uncertain etiology (2), several lines of evidence suggest that a defect in oxidative phosphorylation contributes to its pathogenesis. For instance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that blocks complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain (4), recapitulates in humans the hallmarks of PD (5). Furthermore, reduction in complex I activity has been reported in PD tissues (reviewed in ref. 6). This defect is not confined only to the brain (7), since low complex I activity has also been found in platelets from PD patients (8, 9) and in *cybrid* cells engineered to contain mitochondria derived from platelets of patients suffering from PD (10).

D-β-Hydroxybutyrate (DβHB) is a ketone body produced by hepatocytes and, to a lesser extent, by astrocytes (11). It is an alternative source of energy in the brain when glucose supply is depleted such as during starvation (12). In vitro DβHB prevents neuronal damage seen following glucose deprivation (13) and mitochondrial poison exposure (14). Herein, we show that DβHB infusion protects SNpc dopaminergic neurons against MPTP in a dose-dependent and stereospecific manner and prevents the development of PD-like motor abnormalities in mice. We also provide in vivo and in vitro evidence that DβHB protects not by alleviating MPTP-related complex I inhibition, but by enhancing oxidative phosphorylation via a mechanism dependent on mitochondrial complex II (succinate-ubiquinone oxidoreductase).

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Nonstandard abbreviations used: Parkinson disease (PD); substantia nigra pars compacta (SNpc); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); D-β-hydroxybutyrate (DβHB); L-B-hydroxybutyrate (LβHB); 3-nitropropionic acid (3-NP); tyrosine hydroxylase (TH); 1-methyl-4-phenylpyridinium (MPP^+); carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); transmembrane potential ($\Delta\psi_m$); arbitrary fluorescence unit (AFU); dihydroxyphenylacetic acid (DOPAC); homovanillic acid (HVA); reactive oxygen species (ROS); tricarboxylic acid (TCA).

Methods

Animals and treatment. All animals were 8- to 10-week-old male C57BL mice (Charles River Laboratories, Wilmington, Massachusetts, USA). Mice were divided into four groups: vehicle (i.e., saline), DβHB, L-hydroxybutyrate (LβHB), and DβHB plus 3-nitropropionic acid (3-NP). Vehicle, DβHB (1.6, 0.8, or 0.4 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich, St. Louis, Missouri, USA), and LβHB (1.6 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich) were administered subcutaneously (1 μl/h) using Alzet mini-osmotic pumps (DURECT Corp., Cupertino, California, USA). 3-NP (Sigma-Aldrich;

15 mg/kg, in 0.1 M PBS adjusted to pH 7.4) was given intraperitoneally 2 hours before the implantation on the first day and then once a day until the animals were sacrificed. This dosage of 3-NP was selected to inhibit complex II but not to induce cell loss. After surgery, animals were allowed to rest for 1 day. Each mouse was then randomly assigned to receive four intraperitoneal injections of either MPTP (18 mg/kg of free base in saline; Sigma-Aldrich) or saline at 2-hour intervals.

Tyrosine hydroxylase immunostaining and quantitative morphology. Seven days after the last MPTP injection, mice were killed and their brains were processed for immunohistochemical studies. Sections (30 μm) were incubated with a polyclonal anti-tyrosine hydroxylase (TH; 1,000 dilution; Calbiochem-Novabiochem Corp., San Diego, California, USA) for 48 hours at 4°C. Biotinylated secondary antibodies followed by avidin-biotin complex were used. Immunoreactivity was visualized by incubation in 3,3′-diaminobenzidine/glucose/glucose oxidase. Total numbers of TH-positive neurons in SNpc were counted stereologically using the optical fractionator method (15). Striatal OD of TH immunostaining, determined by the Scion Image program (Scion Corp., Frederick, Maryland, USA), was used as an index of striatal density of TH innervation (16). The concentration of anti-TH antibody and 3,3′-diaminobenzidine (DAB) and the duration of incubation of striatal sections in DAB were optimized to fall within the linear range of the plot of the immunostaining intensities and the scanned ODs.

Measurement of DβHB and succinate levels. At different time points after the implantation of the osmotic pumps, blood was collected from tails, and brains were quickly removed, freeze-clamped under liquid nitrogen, and stored at –80°C. Frozen tissues were treated with perchloric acid and neutralized with sodium hydroxide as previously described (17). Both DβHB and succinate were measured spectrophotometrically at 340 nm using commercial kits from Sigma-Aldrich and from Roche Molecular Biochemicals (Indianapolis, Indiana, USA) respectively, following the manufacturers' instructions.

Measurement of striatal 1-methyl-4-phenylpyridinium levels. Mice infused with either saline or DβHB (1.6 mmol/kg/d) were injected with MPTP (18 mg/kg) as described above and killed 90 minutes after the fourth injection. HPLC with UV detection (295 nm) was used to measure striatal 1-methyl-4-phenylpyridinium (MPP^+) levels as previously described (18) with the following modifications: a reverse-phase Altima C18 column (Alltech Associates Inc., Deerfield, Illinois, USA) and a mobile phase consisting of 89% 50 mM KH_2PO_4 and 11% acetonitrile were used. Data represent mean ± SEM of five mice per group.

Synaptosomal uptake of MPP^+. Striata were dissected out from naive mice and processed for uptake experiments as described previously (19) with a few modifications. Briefly, striata were homogenized in 0.32 M sucrose and centrifuged at 700 *g*, 4°C, for 10 minutes. The supernatant was removed and centrifuged at 27,000 *g* for 30 minutes. The resulting synaptosomal pellet was suspended at 1.2 mg/ml (original wet weight) in Krebs-Ringer phosphate buffer (pH 7.4). The uptake reaction was initiated by addition of 0.6 mg of synaptosomes to tubes containing [^{3}H]MPP^+ (~4 nM, ~800,000 degradations per minute, specific activity 31.6 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA) in the absence or presence of DβHB (up to 5 mM) at 37°C for 6 minutes. Nonspecific uptake was assessed in the presence of 10 μM mazindol. Data represent mean ± SEM of three mice per group.

Isolation of brain mitochondria. Brains from C57BL mice were homogenized in isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, and 2 mg/ml fat-free BSA) using a motorized Dounce homogenizer with eight up-and-down strokes. The homogenate was centrifuged at 1,000 *g* for 10 minutes, and the resulting supernatant was layered onto 5 ml of 7.5% Ficoll medium on top of 5 ml of 10% Ficoll medium and centrifuged at 79,000 *g* for 30 minutes (the Ficoll medium contained 0.3 M sucrose, 50 μM EGTA, and 10 mM HEPES). The mitochondrial pellet was resuspended in isolation buffer. Protein concentrations were determined by the bicinchoninic assay (Pierce Chemical Co., Rockford, Illinois, USA) method with BSA as a standard protein.

Mitochondrial accumulation of MPP^+. Brain mitochondria were isolated and resuspended in buffer as described previously (20) but with a few modifications. The uptake reaction was initiated by addition of 0.6 mg of mitochondria to tubes containing 5 μM [^{3}H]MPP^+ and 45 μM MPP^+ in the absence or presence of DβHB (up to 5 mM) at 25°C for 3 minutes. Nonspecific uptake was assessed in the presence of 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Data represent mean ± SEM of four or five mice per group.

Polarography. Brain mitochondria were suspended in respiration buffer consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM HEPES, 5 mM K_2HPO_4, and freshly added 1 mg/ml defatted BSA at 30°C, and oxygen-consumption rates were measured in a closed-chamber cuvette with a mini-stirring bar using a Clark-type electrode (Hansatech Instruments Ltd., Norfolk, United Kingdom). For each reading, 300 μg protein was used in a final 1-ml respiration buffer, and all mitochondria preparations had an average respiratory control ratio of at least 5 when 10 mM glutamate and 5 mM malate were used as NADH-linked substrates.

ATP measurements. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Where 3-NP was used, it was added from the beginning with MPP^+ (5 minutes) or rotenone (2.5 minutes) to mitochondria before the addition of DβHB. When the reaction was stopped, mitochondrial suspension from the cuvette was lysed in an equal volume of lysis buffer from the ATP biolumi-

nescence assay kit (Roche Molecular Biochemicals), and the content of ATP was measured according to the manufacturer's instructions. Light emitted from luciferase-mediated reaction was captured in a tube luminometer and calculated from a log-log plot of the standard curve of known ATP concentrations.

Measurements of mitochondrial H_2O_2 production. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Phenazine methosulfate (0.1 mM) was used to oxidize NADH (21). Hydrogen peroxide, converted from superoxide by manganese-superoxide dismutase, was measured using 5 μM Amplex red (Molecular Probes, Eugene, Oregon, USA) and 5 U/ml HRP. Fluorescence was detected by a Perkin-Elmer (Boston, Massachusetts, USA) LS55 spectrofluorometer with an excitation wavelength of 550 nm (slit 1.5 nm) and an emission wavelength of 585 nm (slit 3 nm). H_2O_2 production was calculated from a standard curve generated from known concentrations of H_2O_2.

Measurements of mitochondrial transmembrane potential. Safranine, a cationic fluorescence dye accumulated and quenched inside energized mitochondria (22, 23) was used to measure transmembrane potential ($\Delta\psi_m$). Mitochondria were incubated with 10 mM glutamate, 5 mM malate, and 5 μM safranine (Sigma-Aldrich) in respiration buffer 5 minutes before 5 mM DβHB was added, and $\Delta\psi_m$ was monitored for an additional 5 minutes. FCCP (5 μM) was used as a positive control to collapse $\Delta\psi_m$. Fluorescence was detected by a Perkin-Elmer LS55 spectrofluorometer with an excitation wavelength of 495 nm (slit 3 nm) and an emission wavelength of 586 nm (slit 5 nm). Data are reported in arbitrary fluorescence units (AFUs).

Complex I activity. Largely based on protocols described by Birch-Machin and Turnbull (24), brain mitochondria were lysed by freeze-thawing in hypotonic buffer (25 mM KH_2PO_4 [pH 7.2], 5 mM $MgCl_2$) three times. To initiate the reaction, 50 μg mitochondria were added to the assay buffer (hypotonic buffer containing 65 μM ubiquinone$_1$, 130 μM NADH, 2 μg/ml antimycin A, and 2.5 mg/ml defatted BSA) in the absence or presence of different concentrations of rotenone (2.5–15 μM) or MPP^+ (10–30 mM). The oxidation of NADH by complex I was monitored at 340 nm spectrophotometrically for 3 minutes at 30°C prior to the addition of rotenone (2 μg/ml), after which the activity was measured for an additional 3 minutes. The difference in rate before and after the addition of rotenone (2 μg/ml) was used to calculate complex I activity.

Complex II histochemistry. Animals were injected intraperitoneally with either saline or 3-NP (15 mg/kg) once daily for 8 days, the same regimen used in the animals that received DβHB. As described previously (25), animals were perfused with PBS containing 10% glycerol. Brains were rapidly removed, frozen in dry ice–cooled isopentane, and stored at –80°C. Brains were sectioned at 20 μm throughout the entire nigra and striatum. Sections were mounted onto glass microscope slides, and complex II activity was revealed by incubation of sections at 37°C for 20 minutes in 50 mM phosphate buffer (pH 7.6) containing 50 mM succinate as a substrate and 0.3 mM Nitroblue tetrazolium (NBT) as an electron acceptor.

Immunoblots. Total tissue proteins from ventral midbrains of MPTP- and saline-treated mice were isolated as described previously (26), and 20 μg proteins were separated on 12% SDS-PAGE. Membranes were blotted with polyclonal anti-β-hydroxybutyrate dehydrogenase (1:100; a generous gift from Andrew Marks, Columbia University, New York, New York, USA) and monoclonal anti-β-actin (1:5,000) overnight at 4°C. Secondary antibodies conjugated with HRP were used. Bands of interest were analyzed and quantified using FluorChem 8800 (Alpha Innotech Corp., San Leandro, California, USA).

Rotarod performance. The Economex system (Columbus Instruments, Columbus, Ohio, USA), consisting of four rotating rods of 3 cm diameter in separated compartments, enables four mice to be recorded simultaneously. Seven days after MPTP or saline injections, implanted pumps containing 1.6 mmol/kg/d DβHB were removed, and mice (4–13 animals per group) were allowed to recover from surgery and dehydration for an additional 7 days. On the testing day, animals were first pretrained three times (1 hour apart) using an accelerating mode. After these training sessions, the time on the rod, with a maximum recording time of 240 seconds, was recorded for successive rotational speeds (15, 18, 21, 24, 27, 30, 32, 36, and 40 rpm), and the overall rod performance (ORP) for each mouse was calculated by the trapezoidal method as the area under the curve in the plot of time on the rod versus rotation speed (27). To assess the responsiveness of the MPTP-related motor deficit to dopaminergic stimulation, mice were injected intraperitoneally with L-3,4-dihydroxyphenylalanine (L-DOPA) methyl ester/benserazide (100/25 mg/kg), and Rotarod performance was assessed 45 minutes later.

Measurement of dopamine and its metabolite levels in striatal and ventral midbrain tissues. Animals from the Rotarod study were sacrificed, and their striata and ventral midbrains were dissected out and stored at –80°C until analysis. On the day of the assay, striatal and ventral midbrain tissues were sonicated in 50 and 10 volumes (wt/vol), respectively, of 0.1 M perchloric acid containing 50 ng/ml dihydrobenzylamine as internal standard. After centrifugation at 15,000 *g* for 15 minutes at 4°C, 20 μl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA Inc., Bedford, Massachusetts, USA). The mobile phase consisted of 94% 50 mM sodium phosphate/0.2 mM EDTA/1.2 mM heptanesulfonic acid (pH 3.2) solution and 6% methanol. The flow rate was 1.5 ml/min. Peaks were detected by an ESA 8 Channel CoulArray system. Data were collected and processed using the CoulArray data analysis program (version 1.12).

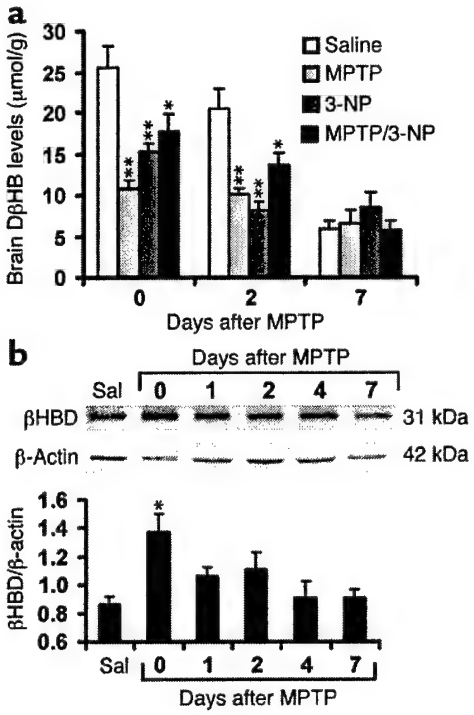

Figure 1
Brain levels of DβHB and β-hydroxybutyrate dehydrogenase (βHBD) under different treatments. (**a**) One day after implantation of pumps containing DβHB, animals were injected intraperitoneally with saline (Sal), MPTP, or 3-NP as described in Methods, and brain levels of DβHB were measured at 0 days (90 minutes after the fourth injection), 2 days, and 7 days thereafter. The utilization of DβHB was increased when cells were under metabolic stress induced by these toxins. $n = 4$–6; $^*P < 0.05$ and $^{**}P < 0.01$ compared with the respective control saline groups. (**b**) Western blot analysis of ventral midbrains from MPTP-intoxicated mice shows upregulation of this enzyme as early as day 0. $n = 4$–5 per group; $^*P < 0.05$ compared with the control saline group. β-Actin is used to normalize βHBD values.

Statistical analysis. All values are expressed as mean ± SEM. Differences between means were analyzed using one-way ANOVA followed by Newman-Keuls post-hoc testing for pairwise comparison. The null hypothesis was rejected when P was greater than 0.05.

Results

MPTP upregulates DβHB-metabolizing enzyme and increases utilization of DβHB in the brain. To assure sustained high tissue levels of DβHB during the experiment, this short-half-life (28) compound was infused subcutaneously at a dose of 1.6 mmol/kg/d for the entire 7 days. This regimen seemed well tolerated and yielded a stable plasma level of approximately 0.9 mM throughout the 7-day period. Likewise, brain DβHB levels in mice intoxicated with MPTP did not significantly change throughout the experiment (Figure 1a). Brain DβHB levels in mice that received saline instead of receiving MPTP, 3-NP, or both were significantly higher, at least at the beginning of the experiment (Figure 1a).

Circulating DβHB readily crosses the blood-brain barrier and enters mitochondria, where it is metabolized by β-hydroxybutyrate dehydrogenase to acetoacetate; the latter is converted to acetyl-CoA, which feeds into the Krebs cycle (29). In saline-injected control mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain (the brain region that contains the SNpc) was detectable (Figure 1b). In MPTP-injected mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain rose rapidly and remained elevated for 2 days after the last injection of MPTP (Figure 1b). These data suggest that MPTP-related cellular stress is associated with a β-hydroxybutyrate dehydrogenase upregulation, which in turn may facilitate utilization of DβHB in the brain.

DβHB attenuates MPTP-induced dopaminergic neurodegeneration. One day after implantation of pumps containing either vehicle or DβHB, mice were injected with MPTP. Seven days later, the brains of these animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. In saline-injected mice infused with either vehicle or DβHB, numbers of TH-positive neurons in the SNpc were identical (Table 1; Figure 2, a and b), as were TH ODs in the striatum (Table 1; Figure 2, i and j). In MPTP-injected mice infused with vehicle, there was an approximately 70% loss of SNpc TH-positive neurons and an approximately 90% reduction of striatal TH ODs (Table 1; Figure 2, e and m) compared with saline-injected controls (Table 1; Figure 2, a and i). In contrast, in MPTP-injected mice

Table 1
TH- and Nissl-positive neurons in SNpc and striatal TH density

	MPTP	3-NP	Nigral TH	Nigral Nissl	Striatal TH OD (×100)
Vehicle	–	–	9,770 ± 694	15,525 ± 930	21.78 ± 1.90
DβHB (1.6 mmol/kg/d)	–	–	9,293 ± 590	14,880 ± 416	23.76 ± 2.10
LβHB (1.6 mmol/kg/d)	–	–	9,040 ± 705	12,987 ± 1,274	20.47 ± 1.43
Vehicle	–	+	8,933 ± 1,040	12,387 ± 1,169	23.11 ± 4.43
Vehicle	+	–	3,233 ± 280	6,445 ± 380	1.61 ± 0.16
Vehicle	+	+	2,600 ± 654	5,860 ± 850	1.76 ± 0.10
DβHB (0.4 mmol/kg/d)	+	–	3,168 ± 625	5,392 ± 847	1.80 ± 0.12
DβHB (0.8 mmol/kg/d)	+	–	3,720 ± 185	7,693 ± 659	2.00 ± 0.39
DβHB (1.6 mmol/kg/d)	+	–	6,300 ± 506[A]	9,597 ± 601	3.73 ± 0.10[B]
LβHB (1.6 mmol/kg/d)	+	–	2,780 ± 236	7,525 ± 360	1.10 ± 0.33
DβHB (1.6 mmol/kg/d)	+	+	1,947 ± 389	4,627 ± 701	1.73 ± 0.27

Animals with pumps containing either vehicle (saline) or different isoforms of β-hydroxybutyrate were injected intraperitoneally with MPTP, 3-NP, or saline (not shown). Data represent mean ± SEM of six to nine mice per group. [A]$P < 0.01$ and [B]$P < 0.05$ compared with the saline-MPTP group.

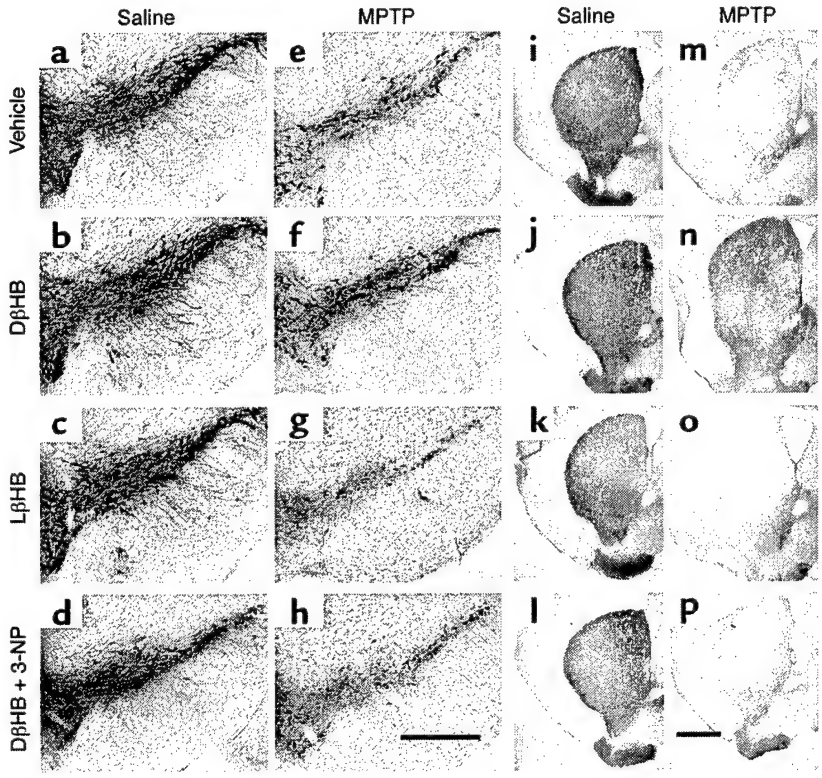

Figure 2
Protective effect of DβHB against MPTP-induced neurodegeneration. (**a–h**) TH-positive neurons in SNpc, and (**i–p**) TH-positive terminals in striatum. Animals were infused subcutaneously with vehicle (saline; **a**, **e**, **i**, and **m**), DβHB (1.6 mmol/kg/d; **b**, **d**, **f**, **h**, **j**, **l**, **n**, and **p**), or LβHB (1.6 mmol/kg/d; **c**, **g**, **k**, and **o**) 1 day before receiving intraperitoneal injections of either saline (**a–d** and **i–l**) or MPTP (18 mg/kg; **e–h** and **m–p**). There is an extensive loss of TH-positive neurons (**e**) and terminals (**m**) in MPTP-injected animals. This loss is attenuated by DβHB (**f** and **n**) but not by its inactive isomer LβHB (**g** and **o**). The complex II inhibitor 3-NP was given intraperitoneally (15 mg/kg) daily for the entire period of DβHB infusion. In the presence of 3-NP, DβHB does not confer neuroprotection. Scale bars: 500 μm (**a–h**) and 1 mm (**i–p**). Please refer to Table 1 for quantification of neurons and terminals in each animal group.

infused with DβHB, less reduction in SNpc TH-positive neurons and striatal TH ODs was observed (Table 1; Figure 2, f and n). To control for the specificity of DβHB neuroprotection, another set of MPTP-injected mice received infusion of the inactive isomer LβHB. In these mice, the loss of dopaminergic neurons was as severe as in mice infused with vehicle (Table 1; Figure 2, g and o). Thus, DβHB, but not its inactive isomer, can attenuate neurotoxic effects of MPTP on dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

DβHB attenuates the loss of dopamine and the motor deficit induced by MPTP. To examine whether DβHB protects not only against structural damage but also against functional deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in ventral midbrain and striatum, as well as locomotor activity, in these animals. In MPTP-injected mice that did not receive DβHB, there was a reduction in dopamine and its metabolites (Table 2) in both ventral midbrain and striatum. Behaviorally, the length of time that these MPTP-injected mice remained on the rotating rods was significantly shorter than that of the saline-injected controls (Figure 3). The motor deficit observed in MPTP-treated mice was alleviated by the administration of L-DOPA/benserazide (data not shown), indicating that this motor deficit results from a loss of dopamine. In MPTP-injected mice that did receive DβHB, the levels of dopamine and its metabolites were all significantly higher than those in MPTP-injected mice that did not receive DβHB (Table 2). Of note, the attenuation of MPTP-induced dopamine loss by DβHB was smaller than the attenuation of MPTP-induced SNpc neuronal death by DβHB. Similarly, MPTP-injected mice that received DβHB performed much better on the rotating rods than MPTP-injected mice that did not receive DβHB (Figure 3). Saline-injected mice that received DβHB had similar levels of dopamine and metabolites (Table 2) and simi-

Table 2
Levels of dopamine and its metabolites in ventral midbrain and striatal tissues

	Ventral midbrain levels (ng/mg tissue)			Striatal levels (ng/mg tissue)		
	DA	DOPAC	HVA	DA	DOPAC	HVA
Vehicle	0.32 ± 0.01	0.098 ± 0.003	1.07 ± 0.02	15.81 ± 0.69	0.91 ± 0.06	1.41 ± 0.03
DβHB	0.33 ± 0.02	0.104 ± 0.010	1.09 ± 0.11	16.92 ± 0.53	1.02 ± 0.01	1.40 ± 0.11
Vehicle/MPTP	0.17 ± 0.01	0.046 ± 0.003	0.50 ± 0.03	0.86 ± 0.21	0.10 ± 0.02	0.36 ± 0.05
DβHB/MPTP	0.23 ± 0.01[A]	0.070 ± 0.005[A]	0.71 ± 0.05[A]	2.41 ± 0.45[B]	0.24 ± 0.03[B]	0.64 ± 0.04[A]

Animals from the Rotarod study were killed, and their brains were removed and measured by HPLC for the levels of dopamine and its metabolites. Data represent mean ± SEM of 4–13 mice per group. [A]$P < 0.01$; [B]$P < 0.05$ compared with the MPTP-treated group without DβHB.

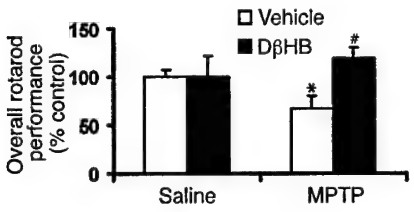

Figure 3
Protective effect of DβHB against motor deficit in MPTP-treated mice. Animals were infused subcutaneously with either vehicle (saline) or DβHB (1.6 mmol/kg/d) 1 day before receiving intraperitoneal injections of either saline or MPTP (18 mg/kg). Pumps were removed at day 7, and animals were allowed to recover from surgery and dehydration for an additional 7 days before their Rotarod performance was assessed. Motor deficit is observed in the MPTP-treated animals, but DβHB significantly improves this impairment. DβHB does not affect base-line motor function in saline-injected mice. $n = 4$–13; $^*P < 0.05$ compared with the saline-vehicle group; $^\#P < 0.05$ compared with the MPTP-vehicle group.

lar motor performance (Figure 3) to those of saline-injected mice that did not receive DβHB.

DβHB does not affect MPTP activation. MPTP is a protoxin whose effect correlates with the striatal content of its active metabolite MPP^+ (30). Striatal levels of MPP^+ 90 minutes after the last injection of MPTP did not differ between mice that received DβHB (30.9 ± 1.6 μg/g tissue) or vehicle [26.8 ± 1.3 μg/g tissue; Student's t test with 6 degrees of freedom ($t(6)$) = 1.98; $P = 0.1$]. MPTP-induced dopaminergic neurotoxicity relies on the entry of MPP^+ into dopaminergic neurons via dopamine transporters (31). DβHB did not impair the uptake of [^{3}H]MPP^+ by striatal synaptosomes at concentrations up to 5 mM, which is more than five times the plasma concentration found in DβHB-infused animals (vehicle, 100% ± 2.3% of control; DβHB, 99.1% ± 1.8% of control; $t(6) = 0.3$; $P = 0.8$). Inside dopaminergic neurons, MPP^+ is concentrated within mitochondria by a mechanism that depends on mitochondrial $\Delta\psi_m$ (20). At 5 mM, DβHB did not alter the uptake of [^{3}H]MPP^+ by purified brain mitochondria (vehicle, 100% ± 4.1% of control; DβHB, 93.2% ± 0.5% of control; $t(6) = 1.7$; $P = 0.1$). Thus, it is unlikely that the neuroprotective effect of DβHB in the MPTP model of PD results from alterations in the key MPTP toxicokinetic steps described above.

DβHB increases mitochondrial oxygen consumption. DβHB has been used as a mitochondrial substrate (32, 33). We thus asked whether DβHB could support oxidative phosphorylation in brain mitochondria, and, if so, whether it may rescue mitochondrial respiration depressed by MPP^+-mediated complex I blockade (34). Consistent with DβHB being a mitochondrial substrate, we found that it increased oxygen consumption in a dose-dependent manner (Figure 4, a and b). The effects of DβHB in supporting mitochondrial respiration are stereospecific, since the inactive isomer LβHB failed to improve oxidative phosphorylation (Figure 4c). We also found that DβHB ameliorated oxygen consumption impaired by different concentrations of MPP^+ (Figure 4a) and of another complex I inhibitor, rotenone (Figure 4b). At 25 μM MPP^+ and 25 nM rotenone, which we found to inhibit about 25% of the oxygen consumption in glutamate- and malate-supported mitochondria, DβHB restored completely the oxygen consumption depressed by these inhibitors (Figure 4, a and b). At 100 μM MPP^+ and 100 nM rotenone inhibits more than 90% of the oxygen consumption in glutamate- and malate-supported mitochondrial respiration (data not shown). At these concentrations, DβHB restored completely the oxygen consumption inhibited by MPP^+, but only partially that inhibited by rotenone (Figure 4, a and b).

DβHB does not uncouple mitochondria. To assure that the increase in rate of oxygen consumption induced by DβHB is not an artifact of uncoupled mitochondria, we measured $\Delta\psi_m$. As expected, the uncoupler FCCP at 5 μM collapsed the $\Delta\psi_m$ in isolated mitochondria (FCCP, 419 ± 23 AFUs; no FCCP, 69 ± 2 AFUs). Conversely,

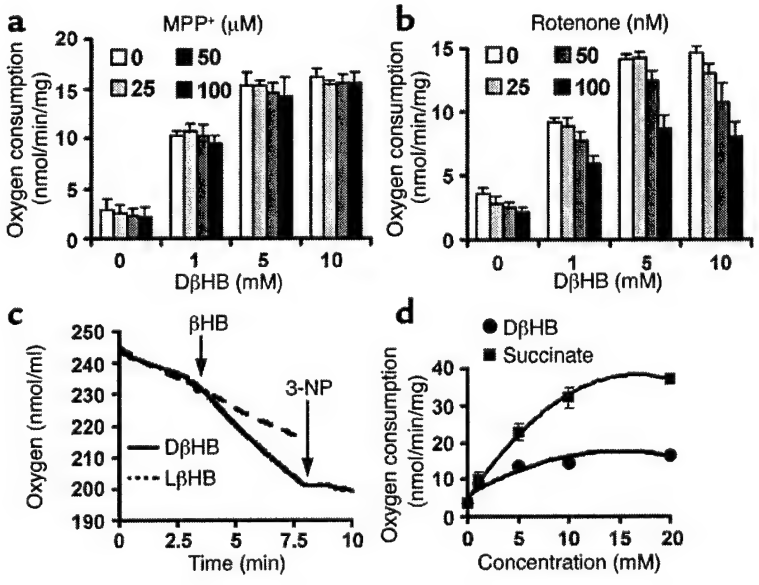

Figure 4
DβHB increases oxygen consumption in purified brain mitochondria. Mitochondria (300 μg) were incubated in the absence or presence of MPP^+ (5 minutes; **a**) or rotenone (2.5 minutes; **b**) at 30°C, and then 5 mM DβHB was added to induce oxygen consumption. DβHB attenuated inhibition of mitochondrial respiration induced by MPP^+ (**a**) or rotenone (**b**) at indicated concentrations, which blocked about 25–90% of oxygen consumption when glutamate and malate were used as NADH-linked substrates (data not shown). (**c**) The improvement of oxygen consumption by DβHB is stereospecific and is blocked by 10 mM 3-NP, a complex II inhibitor. (**d**) DβHB increases oxygen consumption in a dose-dependent and saturable fashion as seen with succinate, a complex II substrate, although not as efficiently as succinate does on an equimolar basis. $n = 3$–4.

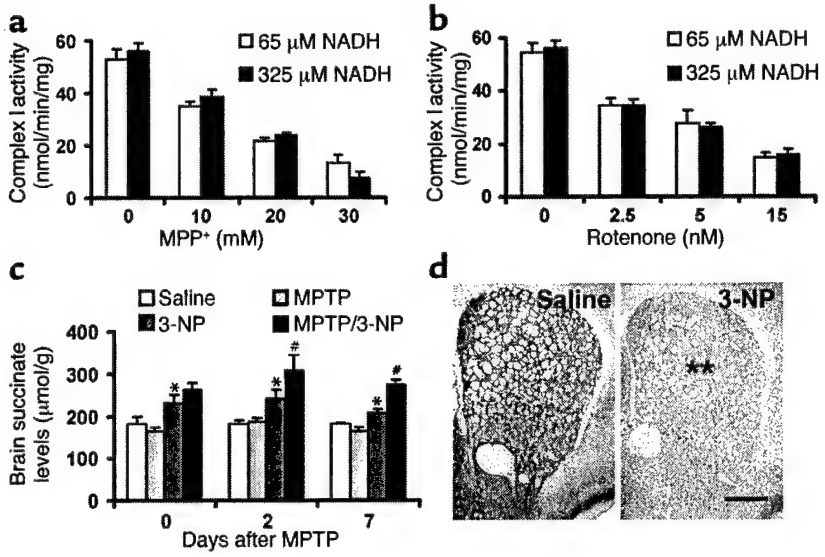

Figure 5

Dose-response study of NADH in complex I activity (**a** and **b**) and brain levels of succinate (**c**). In mitochondria lysed by freeze-thawing, when the inhibition of complex I activity was titrated with different concentrations of MPP^+ (**a**) or rotenone (**b**), different amounts of NADH did not produce different responses in complex I activity ($n = 4$ per group). (**c**) Levels of succinate were measured in the brains of animals treated with 18 mg/kg/d MPTP or 15 mg/kg 3-NP, or both. Levels of succinate in the group that received DβHB (1.6 mmol/kg/d) are significantly increased in the presence of 3-NP. $n = 3$–10 per group; $^*P < 0.05$ compared with the control saline group; $^\#P < 0.05$ compared with the 3-NP group. (**d**) Histochemical analysis in striatal sections shows that when animals were treated with 3-NP (right panel) at this concentration for 8 days, there was approximately 40% reduction in complex II activity in the striatum compared with that in the group treated with saline (left panel). $n = 5$ per group; $^{**}P < 0.01$. Scale bar: 500 μm.

DβHB at concentrations as high as 5 mM had no effect on mitochondrial $\Delta\psi_m$ (DβHB, 68.58 ± 3.07 AFUs; no DβHB, 65.21 ± 3.03 AFUs; $n = 5$ per group; $P > 0.05$). We also found that the increase in oxygen-consumption rate produced by DβHB could be blocked by antimycin A, a complex III inhibitor (base line, 4.49 ± 0.62 nmol/min/mg; DβHB, 14.14 ± 0.43 nmol/min/mg; DβHB + antimycin A, 5.99 ± 0.95 nmol/min/mg; $n = 3$ per group; $P > 0.05$ comparing base line with the DβHB + antimycin A group). These experiments indicate that DβHB does not uncouple mitochondria at concentrations that increased oxygen consumption.

Effects of DβHB on mitochondrial respiration seem driven by complex II. One product generated from the metabolism of DβHB is NADH, which provides the driving force for the mitochondrial respiration through complex I. Can an increase in availability of NADH compensate for the loss of oxygen consumption due to complex I inhibition? To test this possibility, freeze-thawed disrupted brain mitochondria were incubated with MPP^+, or rotenone, and NADH. Concentrations of MPP^+ and rotenone were selected to produce complex I inhibition ranging from about 40% to 100%, and supplementation of NADH ranged from 0.5 to 2.5 times the normal concentration used in the assay (Figure 5, a and b). These changes in NADH supplementation did not modify the degree of complex I inhibition (Figure 5, a and b). This indicates that DβHB-derived NADH cannot explain the improvement seen in mitochondrial respiration produced by DβHB.

Based on its metabolic pathway, DβHB can also generate succinate, which is capable of stimulating the rate of oxygen consumption in isolated brain mitochondria through complex II. In keeping with this metabolic pathway, we found that both DβHB and succinate did improve oxygen consumption in a dose-dependent and saturable manner, although DβHB was not as potent as succinate (Figure 4d). This is not unexpected, since DβHB has to go through several metabolic steps to generate succinate. In addition, we found that the beneficial effects of DβHB on mitochondrial respiration in the presence of MPP^+ or rotenone were completely abolished by two different complex II inhibitors, 3-NP at 10 mM (Figure 4c) and malonate at 10 mM (data not shown). Together, these data are consistent with the idea that DβHB increases mitochondrial respiration in the face of complex I inhibition by a complex II–dependent mechanism.

DβHB neuroprotection is abrogated by mitochondrial complex II inhibition in vivo. To determine whether our in vitro data are relevant to DβHB neuroprotection seen in vivo, we first measured succinate levels in the brains of DβHB-infused mice. Upon inhibition of complex II, DβHB infusion indeed increased levels of succinate in the brain (Figure 5c). Next, MPTP-injected mice infused with DβHB were injected with 3-NP. This irreversible complex II inhibitor was administered daily for the entire period of DβHB infusion at a dosage of 15 mg/kg/d. As illustrated in Figure 5d, this regimen of 3-NP inhibited approximately 40% of complex II activi-

Table 3
ATP levels in purified brain mitochondria

ATP levels (nmol/mg mitochondrial protein)	
Base line (no substrate)	5.37 ± 0.30
DβHB (5 mM)	76.16 ± 6.11[A]
DβHB plus MPP^+ (100 μM)	90.49 ± 9.73[A]
DβHB plus rotenone (100 nM)	25.96 ± 5.22[B]
DβHB plus MPP^+ plus 3-NP (10 mM)	0.62 ± 0.21
DβHB plus rotenone plus 3-NP	0.73 ± 0.23
LβHB	3.85 ± 0.24

Mitochondrial samples were prepared as in the polarographical studies, and ATP levels were measured using a luciferase kit. Data represent mean ± SEM of four mice per group. $^AP < 0.01$ and $^BP < 0.05$ compared with the base-line endogenous ATP level.

Table 4
H_2O_2 measurements in purified brain mitochondria

Treatment	Mitochondrial H_2O_2 production (pmol/min/mg protein)
DβHB (5 mM)	73.83 ± 8.04
Rotenone (100 nM)	132.39 ± 19.68
DβHB plus rotenone	506.00 ± 40.47[A]
DβHB plus rotenone plus 3-NP (10 mM)	522.76 ± 62.23[A]
DβHB plus rotenone plus PM (0.1 mM)	160.50 ± 20.62
LβHB (5 mM) plus rotenone	105.91 ± 7.45
MPP^+ (500 μM)	55.24 ± 12.98
DβHB plus MPP^+	94.92 ± 6.79[B]
DβHB plus MPP^+ plus PM	73.76 ± 6.38
LβHB plus MPP^+	54.28 ± 4.93

Mitochondrial samples similar to those in the polarographical studies were prepared, and the fluorescence dye Amplex red was used to measure H_2O_2 converted from superoxide. Data represent mean ± SEM of four mice per group. [A]$P < 0.01$ compared with the rotenone-alone group; [B]$P < 0.05$ compared with the MPP^+-alone group. PM, phenazine methosulfate.

ty in the striatum without causing cell death in either the SNpc (Table 1) or the striatum, as evidenced by TH or Nissl staining (Table 1; data not shown for striatal Nissl staining). As before, DβHB protected against MPTP neurotoxicity in mice that did not receive 3-NP. However, DβHB failed to reduce MPTP-induced dopaminergic neurodegeneration in mice that did receive 3-NP (Table 1; Figure 2, h and p). Supporting the effectiveness of the 3-NP regimen in blocking complex II is our demonstration that succinate levels in the brain were higher in mice that received 3-NP than in those that did not (Figure 5c). Thus, these results are consistent with the hypothesis that complex II is a pivotal mediator in DβHB's neuroprotective effects.

DβHB does not have antioxidant effects but increases ATP production. Inhibition of complex I by MPP^+ and rotenone generates reactive oxygen species (ROS), raising the possibility that the beneficial effects of DβHB are mediated by an antioxidant action, as previously suggested (14). In isolated mitochondria, DβHB did not reduce but stimulated ROS production in the presence of rotenone or MPP^+ (see Table 4). To elucidate the basis of DβHB-related ROS production, 3-NP was added to the incubation mixture (see Table 4). This complex II inhibitor was unable to block the DβHB-related ROS production, thus ruling out the possibility of a reversed flux of electrons from complex II to complex I as the ROS generator (22, 23). Instead, we suspected that the DβHB-related ROS resulted from additional NADH generated by DβHB metabolism. To test this alternative possibility, phenazine methosulfate, a compound that oxidizes NADH (21), was included in the incubation mixture. Consistent with this possibility, phenazine methosulfate abolished ROS production (see Table 4). These data argue against DβHB having antioxidant properties, at least in this in vitro setting.

Inhibition of complex I by MPP^+ and rotenone also impairs ATP production, raising the possibility that the beneficial effects of DβHB are mediated by attenuation of ATP depletion. We thus measured ATP production in isolated brain mitochondria under conditions similar to those of polarographical study. As shown in Table 3, DβHB increased ATP production from a base line of 5.37 ± 0.30 nmol/mg protein to 76.16 ± 6.11 nmol/mg protein. The increase of ATP production was not detected with the inactive isomer LβHB (3.85 ± 0.24 nmol/mg protein). In agreement with the oxygen-consumption data, DβHB prevented the loss of ATP production caused by 100 μM MPP^+ or 100 nM rotenone (Table 3). Yet, upon addition of 3-NP, DβHB-related ATP production was abolished (Table 3). Together, these data are consistent with the contention that the effects of DβHB seen in the polarographical studies correspond to an increase in oxidative phosphorylation.

Discussion

The present study shows that the ketone body DβHB, a crucial alternative source of glucose for brain energy, confers protection against the structural and functional deleterious effects of the parkinsonian toxin MPTP; these include degeneration of SNpc dopaminergic neurons and striatal dopaminergic fibers, loss of striatal dopamine, and PD-like motor deficit. The beneficial effects of DβHB were achieved by its subcutaneous infusion using osmotic mini-osmotic pumps, which, without apparent distress, allowed its reliable continuous delivery to the brain. While DβHB levels in the brain were stable in DβHB-infused mice exposed to MPTP, in mice injected with saline they were higher at the beginning and then dropped during the experimental period of 7 days. Although the basis for these differences remains to be elucidated, it is possible that the utilization of DβHB in the brain increases rapidly following exposure to mitochondrial poisons such as MPTP and augments progressively in normal brain as part of a metabolic adaptation to sustained high DβHB concentrations.

Utilization of DβHB in the brain is contingent on its conversion to acetoacetate by β-hydroxybutyrate dehydrogenase, which is scarce in the adult brain, especially in the basal ganglia (35). The activity of β-hydroxybutyrate dehydrogenase correlates with its protein content (36), and, following MPTP administration, it is upregulated in the ventral midbrain. MPTP-induced β-hydroxybutyrate dehydrogenase upregulation precedes peak dopaminergic neuronal death in this model (37). It can thus be envisioned that β-hydroxybutyrate dehydrogenase activity increases early enough to allow effective utilization of DβHB by the compromised dopaminergic neurons.

A critical step in activation of MPTP is its conversion into MPP^+ by monoamine oxidase (38). The possibility that DβHB infusion confers protection by interfering with monoamine oxidase activity can be ruled out given the fact that brain levels of MPP^+ were similar between mice that received and those that did not receive DβHB. Also arguing against the possibility that DβHB confers protection by impairing MPTP activation is the fact that DβHB attenuates dopaminergic neuronal death in primary ventral midbrain cultures exposed to MPP^+ (14). DβHB also did not interfere with other key aspects of

MPTP metabolism (39), such as entry of MPP^+ into dopaminergic neurons and mitochondria at concentrations as high as 5 mM. Together these data indicate that DβHB protects not by a pre–complex I mechanism but rather by mitigating the deleterious effects of complex I inhibition on the survival of dopaminergic neurons.

In isolated brain mitochondria, DβHB improves oxygen consumption in the presence of the complex I poisons MPP^+ and rotenone. The DβHB effect is dose dependent and stereospecific. The metabolism of DβHB leads to an elevated mitochondrial [NADH]/[NAD^+] ratio due to NADH generated from the conversion of DβHB to acetoacetate and also from the tricarboxylic acid (TCA) cycle, whose turnover is increased by high levels of acetyl-CoA produced by acetoacetate. NADH is used by complex I to drive mitochondrial respiration. DβHB may increase oxygen consumption by fueling mitochondria with NADH. However, in the presence of complex I inhibition by MPP^+ or rotenone, NADH oxidation is impaired and, as shown in this study, an increase in NADH content is unable to alleviate complex I blockade.

In addition to generating NADH, increased TCA turnover, in theory, should also lead to increases in production of other TCA intermediates such as succinate. Here, we show that DβHB infusion does increase brain succinate content. While succinate is a TCA cycle substrate, its oxidation by succinate dehydrogenase is coupled to a transfer of electrons to ubiquinone of the mitochondrial respiratory chain, and thus succinate is routinely used to support oxygen consumption in the presence of complex I blockade. We demonstrate that inhibition of complex II (a) abrogates DβHB-mediated increases in oxygen consumption in isolated mitochondria and (b) abolishes DβHB-mediated protective effects on SNpc dopaminergic neurons and striatal dopaminergic fibers after MPTP administration. Thus, these data strongly support our hypothesis that the beneficial effect of DβHB in the MPTP model of PD involves a complex II–dependent mechanism.

It has been proposed that the ability of DβHB to decrease MPP^+ neurotoxicity in primary ventral midbrain cultures is related to the oxidation of the coenzyme Q couple, which should, by decreasing the semiquinone, decrease ROS production (14). Contrary to this prediction, we found, at least in isolated mitochondria, that rather than decreasing ROS production induced by MPP^+ or rotenone, DβHB enhanced it even further. These findings cast doubt that DβHB protects the nigrostriatal pathway through an antioxidant mechanism. How can DβHB increase ROS? Succinate is the most effective ROS-generating substrate in intact brain mitochondria (22, 23), by stimulating a reversed flux of electrons from complex II to complex I (22, 23). However, rotenone blocks this ROS signal (22, 23); thus, in the context of the present study, in which complex I is inhibited, this mechanism may not be operative. Instead, our data suggest that DβHB-derived NADH, by feeding complex I, increases the accumulation of electrons upstream to the blockade, thereby stimulating ROS production.

Mitochondrial respiration is tightly linked to ATP synthesis (40). It may thus be speculated that DβHB, by restoring oxygen consumption in MPTP-intoxicated animals, may increase ATP cellular stores. Ablation and inhibition of poly(ADP-ribose) polymerase-1 (41, 42) and creatine supplements (43) mitigate MPTP-induced death of dopaminergic neurons in the SNpc by buffering ATP depletion. These studies underscore the importance of ATP deficit in the MPTP neurodegenerative process. In normal rodents, dopaminergic structures represent less than 15% of the cellular elements in the striatum (44) and hardly more in the ventral midbrain. This renders precarious any detection of ATP changes in brain tissues of MPTP-intoxicated mice (45). To avoid this problem, we studied the effects of DβHB on ATP production in isolated brain mitochondria. By this approach, we were able to demonstrate that DβHB does increase ATP levels in both the absence and the presence of complex I inhibitors. Consistent with the oxygen-consumption data, we also found that the stimulation of ATP production by DβHB likely relies on complex II, as inhibitors of this electron transport chain enzyme eliminated the effect. Data generated in isolated mitochondria may only approximate the more complex situation found in vivo. Despite this caveat, we believe that the most parsimonious explanation for DβHB-induced neuroprotection in the MPTP model of PD is that energy crisis is attenuated by an enhancement of oxidative phosphorylation. It is thus tempting to conclude that, under the current DβHB regimen, the benefit due to the improved ATP production overcomes the possible detriment due to the increased ROS formation in this PD model.

The present study demonstrates that modulation of body DβHB levels may be a straightforward neuroprotective strategy for the treatment of neurodegenerative diseases such as PD. Relevant to this view is the demonstration that mice subjected to dietary restriction (e.g., alternate-day fasting) exhibit higher serum DβHB concentrations and are more resistant to kainic acid–induced hippocampus damage (46) and to MPTP-induced SNpc damage (47). At this point, however, the long-term effects of the chronic use of DβHB on the cell metabolism and, especially, on the mitochondrial function are not known. DβHB has been administered orally for several months to two 6-month-old infants with hyperinsulinemic hypoglycemia (48). Despite the high dosage (up to 32 g/d), these patients seem to tolerate quite well. In addition, the ketogenic diets, which result in high levels of DβHB, have been used for more than 70 years in humans as a treatment for refractory epilepsy and have proven safe and well tolerated.

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L-3-Hydroxyacyl-CoA Dehydrogenase II Protects in a Model of Parkinson's Disease

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The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) impairs mitochondrial respiration and damages dopaminergic neurons as seen in Parkinson's disease (PD). Here, we report that L-3-hydroxyacyl-CoA dehydrogenase type II/amyloid binding alcohol dehydrogenase (HADH II/ABAD), a mitochondrial oxidoreductase enzyme involved in neuronal survival, is downregulated in PD patients and in MPTP-intoxicated mice. We also show that transgenic mice with increased expression of human HADH II/ABAD are significantly more resistant to MPTP than their wild-type littermates. This effect appears to be mediated by overexpression of HADH II/ABAD mitigating MPTP-induced impairment of oxidative phosphorylation and ATP production. This study demonstrates that HADH II/ABAD modulates MPTP neurotoxicity and suggests that HADH II/ABAD mimetics may provide protective benefit in the treatment of PD.

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Parkinson's disease (PD) is a common neurodegenerative disease whose main neuropathological feature is the loss of the substantia nigra pars compacta (SNpc) dopaminergic neurons.[1] Although its cause remains unknown, the mechanism of SNpc dopaminergic neuronal death may involve a defect in oxidative phosphorylation.[1] Indeed, reduction in the activity of complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain has been reported in PD tissues (reviewed in Greenamyre and colleagues[2]), especially in the brain[3] and platelets.[4,5] Supporting the significance of this mitochondrial defect in SNpc neuronal death are the observations that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that blocks complex I,[6] reproduces the hallmarks of PD in humans.[7] Furthermore, alternative brain energy substrates, such as the ketone body D-β-hydroxybutyrate or creatine, by improving oxidative phosphorylation, do attenuate dopaminergic neurodegeneration caused by the parkinsonian toxin MPTP.[8–10]

Type II L-3-hydroxyacyl-CoA dehydrogenase/amyloid β peptide binding alcohol dehydrogenase (HADH II/ABAD) is predominantly a mitochondrial enzyme[11–13] that belongs to the short-chain dehydrogenase/reductase superfamily.[14] HADH II/ABAD catalyzes reversibly the third step of fatty acid β oxidation in mitochondria, converting L-3-hydroxyacyl-CoA in the presence of NAD^+ to 3-ketoacyl-CoA, NADH, and H^+.[15] HADH II/ABAD appears to be a multifunctional enzyme with a broad range of substrates.[16] In a murine cerebral ischemia model, HADH II/ABAD overexpression reduces infarct size and neurological deficit scores presumably by enhancing the flux of acetyl-CoA through the tricarboxylic acid (TCA) cycle and by increasing ATP levels in the brain.[17] Although overexpression of HADH II/ABAD is protective in acute brain injury models,[17] its role in a chronic neurodegenerative process, such as in PD, is not known. In this study, we show that not only is HADH II/ABAD downregulated in the SNpc of both PD patients and MPTP-treated mice, but also its overexpression in transgenic mice attenuates MPTP-induced dopaminergic neurodegeneration and ATP depletion. These results indicate that HADH II/ABAD may contribute to determining the fate of compromised SNpc dopaminergic neurons.

Materials and Methods

Animals and Treatment

Characteristics of the transgenic (Tg) mice with approximately four fold increased expression of HADH II/ABAD in neurons can be found in Yan and colleagues.[17] In these an-

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imals, expression of human HADH II/ABAD is driven by the platelet-derived growth factor B-chain promoter. Male C57/bl transgenic HADH II/ABAD hemyzygotes were mated with female wild-type C57/bl mice to yield transgenic and wild-type littermates. Genotyping by polymerase chain reaction was performed on DNA extracted from mouse tails using the following pair of primers: 5′-AGGGCAGAGGAGCGTGTGT-3′ (forward) and 5′-GGCAGCAGCGTGTCGGAGCG-3′ (reverse) and polymerase chain reaction amplification conditions: denaturation at 95°C for 20 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute for 30 cycles. Eight- to 10-week-old mice received one intraperitoneal injection of MPTP-HCl (30mg/kg/day of free base) or saline for 5 consecutive days and were killed at 0, 2, 4, 7, and 21 days after the last injection.

Immunostaining and Quantitative Morphology in Mouse Samples

Twenty-one days after the last MPTP injection, mice were killed and their brains were processed for immunohistochemical studies following our standard protocol,[9] using a polyclonal anti-tyrosine hydroxylase (TH) (1:1,000; Calbiochem, San Diego, CA) or a monoclonal anti-HADH II/ABAD (1:50).[18] Total numbers of SNpc TH-positive neurons were counted using the stereological optical fractionator method.[19] Striatal optical density (OD) of TH fibers[9] was determined using the Scion Image program. Colocalization of HADH II/ABAD with TH was studied using double immunofluorescence followed by confocal microscopic analyses in naive mice.

Quantification of Apoptotic Cells

Four days after MPTP injections, mice were killed and their brains were processed for TH immunostaining as described above and were counterstained with thionin for Nissl substances. Total numbers of apoptotic cells were counted as previously described.[20,21]

Immunoblots

Tissue proteins from ventral midbrains of MPTP- and saline-treated mice and of human brains were isolated[21] and immunoblotted[18] using primary monoclonal antibodies to either HADH II/ABAD (1:15,000), β-actin (1:1,500; Sigma-Aldrich, St. Louis, MO), or polyclonal anti-TH (1:1,000). To assess the translocation of HADH II/ABAD from the matrix to the mitochondrial membrane, brain mitochondria from naive mice were prepared as for the polarographical studies (see below). All mitochondrial preparations had an average respiratory control ratio of ≥ 5. At the end of the incubation period with different substrates and inhibitors, potassium thiocyanate (150mM final concentration) was added to the mixture to stabilize the bound complex,[22] before they were freeze-thawed five times in liquid nitrogen. The samples were then centrifuged at 150,000*g* 1 hour, and the pellet was resuspended in the assay buffer. Mitochondrial membrane protein was immunoblotted using a primary antibody to either HADH II/ABAD (1:15,000), cytochrome oxidase (COX; subunit IV; 1:1,1000; Molecular Probes, Eugene, OR), or heat shock protein-60 (HSP-60, 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of Striatal Dopamine and Its Metabolites

Twenty-one days after the last MPTP injection, striatal levels of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured by high-performance liquid chromatography with electrochemical detection as described previously.[9]

MPTP Metabolism, Uptake of MPP^+, and Measurement of Lactate Levels

Striatal MPP^+ levels, synaptosomal uptake of [^{3}H]-MPP^+ and mitochondrial accumulation of [^{3}H]-MPP^+ were determined as described previously.[9,23] Lactate production was measured in mouse striatal sections in the presence of MPP^+ as described previously.[23]

Polarography

Oxygen consumption in brain mitochondria was monitored as described[9] using a Clark-type electrode (Hansatech Instruments, PP System, Haverhill, MA). To assess mitochondrial respiration mediated through complex I, we preincubated mitochondria with 10mM glutamate and 5mM malate in the absence or presence of MPP^+ (5 minutes) or rotenone (2.5 minutes) before 500μM ADP was added to induce state 3 respiration. For complex II– (succinate-ubiquinone oxidoreductase) mediated respiration, mitochondria were preincubated for 2.5 minutes with 10mM succinate and 1μM rotenone in the absence or presence of the complex II inhibitor malonate before 500μM ADP was added. For complex IV– (cytochrome oxidase) mediated respiration, mitochondria were preincubated for 2.5 minutes in the absence or presence of the complex IV inhibitor potassium cyanide before 10mM ascorbate and 0.2mM N,N,N′,N′-tetramethyl-benzendiamine (Cayman Chemical, Ann Arbor, MI) were added to induce oxygen consumption.

Mitochondrial ATP and Hydrogen Peroxide Measurements

Samples were prepared under the identical conditions as those for the polarographical studies. ATP was measured using a luciferase luminometric assay as previously described.[9] Hydrogen peroxide, converted from superoxide by manganese-superoxide dismutase, was measured using 5μm Amplex Red and 5U/ml horseradish peroxidase as described previously.[9]

Activities of Mitochondrial Respiratory Chain Complexes in the Mouse Brain

The measurements of activities of mitochondrial complexes I, II, and IV in both brain mitochondria and brain homogenates were performed as previously described[9,24] with several modifications. In brief, freeze-thawed lysed samples (50μg) were used for complex I and II assays. For complex I activity, the oxidation of NADH (EC = $6.23 mM^{-1}cm^{-1}$) by complex I was monitored at 340nm. For complex II activity, the reduction of dichlorophenolindophenol was monitored at 600nm (EC = $19.1 mM^{-1}cm^{-1}$). For complex IV activity, samples (10μg) were added to assay medium (20mM KH_2PO_4, 30mM *n*-dodecyl-β-D-maltoside and 1%

reduced cytochrome c) to initiate the reaction. The oxidation of cytochrome c was measured as the initial rate at 550nm (EC = 19.1mM^{-1}cm^{-1}). Reduced cytochrome c, used as a substrate for complex IV, was prepared fresh by adding a few grains of sodium hydrosulfide as previously described[25] such that the absorbance was in the range of 1.8 to 2.0.

Citrate Synthase Activity

Samples (10μg) were added to the assay medium (0.1M Tris-HCl, pH 8.1, containing 1.0mM 5,5′-dithiobis-[2-nitrobenzoic acid], 10mM acetyl-CoA, and 0.1% Triton X-100) at 30°C. Freshly prepared 10mM oxaloacetate was added to initiate the reaction. The reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) was monitored at 412nm (EC = 13.6mM^{-1}cm^{-1}).

Immunostaining of Human Samples

Human tissue samples were obtained from the Parkinson Brain Bank at Columbia University. Age at death and interval from death to tissue processing were 72.2 ± 8.8 years and 13.0 ± 3.5 hours (mean ± SEM), respectively, for the control group (n = 10) and 77.2 ± 2.3 years and 10.1 ± 2.4 hours, respectively, for the PD group (n = 11). Paraffin-embedded sections (7μm) were deparaffinized and microwaved in 10mM citrate buffer (pH 6.0) for antigen retrieval before incubation with the anti-HADH II/ABAD (1:50). As a negative control, anti–HADH II/ABAD antibody was preabsorbed with excess recombinant HADH II protein (50μg/ml, generated as described in Yan and colleagues[17]). Immunostaining was visualized using 3,3′-diaminobenzidine with cobalt/nickel enhancement.

Statistical Analysis

All values are expressed as mean ± SEM. One-way or two-way analysis of variance was used (unless indicated otherwise) followed by Newman–Keuls post hoc testing for pairwise comparison. The null hypothesis was rejected at the 0.05 level.

Results

HADH II/ABAD Protein Levels Are Reduced in Postmortem Samples from Parkinson's Disease Patients

Although HADH II/ABAD has been shown to be expressed ubiquitously in human brains,[18,26] to assess the relevance of HADH II/ABAD in PD, we asked whether this enzyme is indeed expressed in SNpc dopaminergic neurons, and whether its expression level is altered by the disease process. Using Western blot analyses, we detected a significant reduction in HADH II/ABAD levels in postmortem ventral midbrain samples from PD patients as compared with those from the control subjects (Fig 1A); however, in striatal and cerebellum tissues, HADH II/ABAD levels did not differ between the two groups (data not shown).

Immunohistochemical studies confirmed the ubiquitous expression of HADH II/ABAD in human neurons (not shown), including SNpc dopaminergic neurons, which are readily identifiable by their content of the brown pigment neuromelanin (see Fig 1B–F). Also apparent was a consistently fainter HADH II/ABAD immunoreactivity (blue-gray) in SNpc dopaminergic neurons in PD patients (see Fig 1D, F) compared with the control subjects (see Fig 1C, E). HADH II/ABAD immunostaining also was detected in the core of the prototypical PD proteinacious intraneuronal inclusions, Lewy bodies (see Fig 1D, arrow). These findings indicate that HADH II/ABAD is present in both normal and diseased SNpc dopaminergic neurons and that its expression is reduced in these ventral midbrain neurons from PD patients.

HADH II/ABAD Is Also Reduced in Mouse Ventral Midbrain after MPTP Injection

To assess whether the reduction in HADH II/ABAD seen in PD tissues is not simply a nonspecific alteration due to dying neurons, we turned to the MPTP mouse

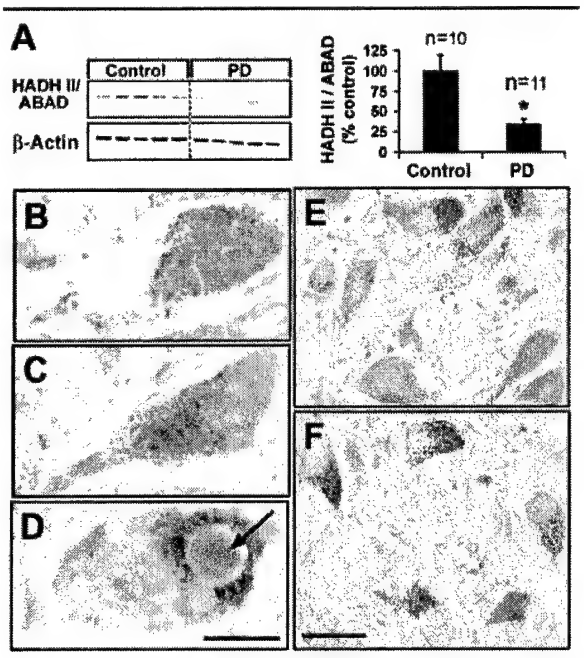

Fig 1. Reduction of HADH II/ABAD levels in Parkinson's disease (PD) patients. Western blot analyses (A) of postmortem ventral midbrain samples show PD patients have significantly lower levels of HADH II/ABAD than control subjects. HADH II/ABAD levels were quantified as the ratio of HADH II/ABAD over β-actin and expressed as percentage of control. Immunohistochemical studies confirm the reduction of this protein in dopaminergic neurons (B–F; brown, neuromelanin; blue-gray, HADH II/ABAD) of PD patients (D, F; arrow *in D: Lewy body) as compared with those of the control subjects (C, E). As a negative control, HADH II/ABAD recombinant protein was added to preabsorb the HADH II/ABAD antibody (B). Data represent mean ± SEM of 10 (control) and 11 (PD) subjects per group,* (asterisk) $p < 0.05$ *compared with control subjects. Scale bars: 25μm (B–D) and 50μm (E, F).*

model of PD. As found in control human samples, mouse ventral midbrain sections immunostained for HADH II/ABAD showed this enzyme is expressed ubiquitously in cells exhibiting essentially a neuronal morphology, including those in the SNpc (Fig 2A–D). By confocal microscopy, HADH II/ABAD immunoreactivity (see Fig 2B) was demonstrated to colocalize (see Fig 2D) with TH (see Fig 2C), a marker for dopaminergic cells. Thus, these results indicate that HADH II/ABAD is highly expressed in mouse neurons including SNpc dopaminergic cells.

To assess ventral midbrain, striatal, and cerebellar levels of HADH II/ABAD during the neurodegenerative process, we performed time course studies in MPTP-injected mice. Like in human samples, ventral midbrain expression of HADH II/ABAD, assessed here by its tissue protein contents (see Fig 2E), was reduced in MPTP-injected mice compared with saline-injected controls. In contrast with HADH II/ABAD, TH levels were not significantly reduced under this MPTP regimen, indicating that the reduction of HADH II/ABAD was not secondary to the loss of TH neurons. The nadir of ventral midbrain HADH II/ABAD protein contents occurred between 2 and 7 days after MPTP injections (see Fig 2E), a period of time that corresponds to the most active phase of apoptosis in SNpc seen with this MPTP regimen.[21] Thereafter, HADH II/ABAD levels progressively increased back to control levels. In contrast with the ventral midbrain region, HADH II/ABAD protein levels did not differ between MPTP- and saline-injected mice in the cerebellum and in the striatum (data not shown).

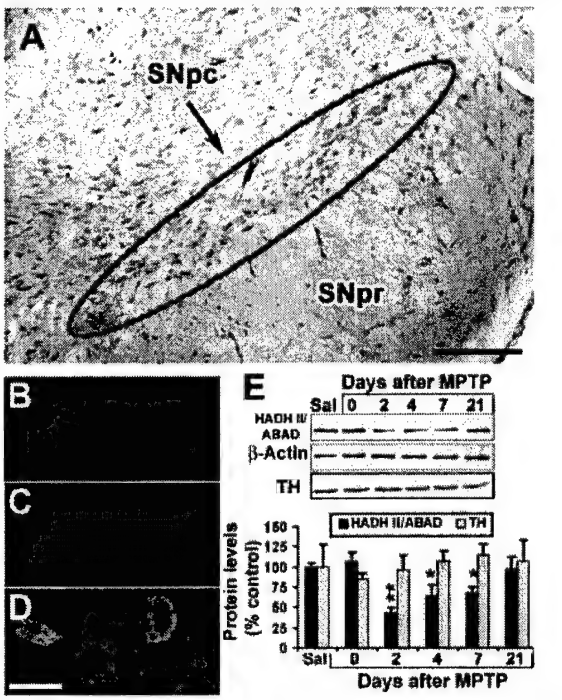

Fig 2. Expression of HADH II/ABAD in substantia nigra pars compacta (SNpc) tyrosine hydroxylase (TH)-positive neurons and its reduction in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–treated mice. Immunoreactivity of HADH II/ABAD is highly abundant in mouse ventral midbrain and the region of interest, substantia nigra (A). Analyses of double immunofluorescence confirm that HADH II/ABAD (B, green) colocalizes with TH-positive neurons (C, red) as demonstrated when they are merged (D, yellow). Scale bars = 250μm (A) and 25μm (B–D). In ventral midbrain samples, MPTP reduces levels of HADH II/ABAD but not TH proteins (E) at indicated time points. Protein levels were quantified as the ratio of HADH II/ABAD or TH over β-actin and expressed as percentage of their respective controls. Data represent mean ± SEM of 6 to 10 mice per group. (single asterisk) $p < 0.05$ *and* (double asterisks) $p < 0.01$ *compared with the control saline group.*

HADH II/ABAD Protects against MPTP-Induced Neurodegeneration

In light of the MPTP-induced SNpc HADH II/ABAD downregulation, we assessed the role of this enzyme in MPTP neurotoxicity by comparing its toxic effects in transgenic mice overexpressing HADH II/ABAD specifically in neurons and in their wild-type littermates. In saline-injected mice, no difference in dopaminergic neurons was detected between the two groups of animals (Fig 3A, C, I). MPTP caused a marked loss of SNpc TH-positive neurons in wild-type mice (see Fig 3B, I), but not as drastic a loss as in Tg-HADH II/ABAD mice (see Fig 3D, I). Similarly, there were fewer MPTP-induced SNpc apoptotic neurons in Tg-HADH II/ABAD mice than in their wild-type counterparts (see Fig 3K, L).

Sparing of SNpc dopaminergic neurons does not always correlate with sparing of their corresponding striatal nerve fibers,[19] which is essential for maintaining dopaminergic neurotransmission. As seen in SNpc neuronal counts, the striatal optical density of TH immunostaining (see Fig 3E–H) in MPTP-injected mice was higher in Tg-HADH II/ABAD mice than in their wild-type littermates (see Fig 3J). In contrast, there were no differences in the extent of decline of striatal levels of dopamine and its main metabolites, DOPAC and HVA, between genotypes, after MPTP administration (Table 1). These findings indicate that HADH II/ABAD overexpression protects the nigrostriatal pathway structurally but not functionally against the toxicity of MPTP.

HADH II/ABAD Does Not Affect MPTP Activation

Striatal levels of MPP^+ 90 minutes after MPTP injection did not differ between Tg-HADH II/ABAD mice (29.26 ± 5.77μg/g striatal tissue, n = 4) and their wild-type littermates (30.40 ± 2.57, n = 6). Lack of difference between genotypes also was observed in the

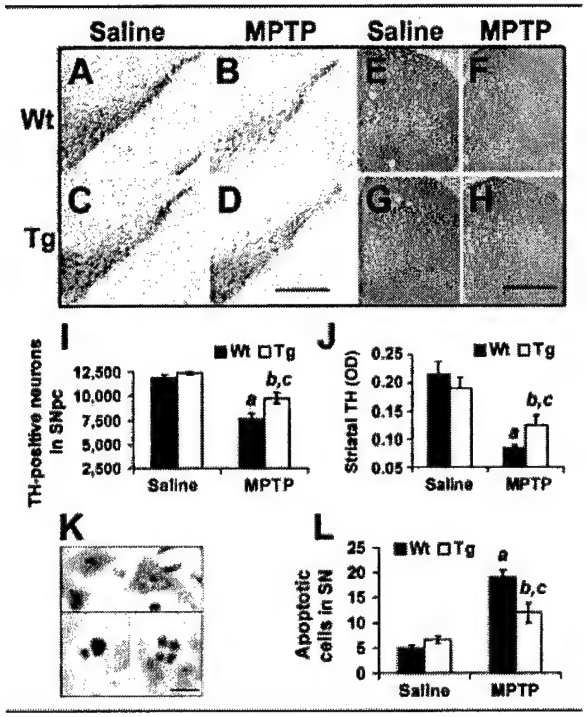

Fig 3. Protective effect of HADH II/ABAD against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced neurotoxicity. Overexpression of HADH II/ABAD significantly attenuates the loss of tyrosine hydroxylase (TH)-positive neurons (D, I) and terminals (H, J) as compared with their wild-type (Wt) counterparts (B, I) and (F, J). HADH II/ABAD also attenuates apoptotic cell death in substantia nigra (L) as evidenced by Nissl staining (K). Compared with normal neurons (K, top panel), these apoptotic cells appear as distinct, round, and well-defined cells as a result of chromatin condensation and shrinkage of cell body (K, bottom panels). Data represent mean ± SEM of three to six mice per group. $^{a}p < 0.001$ *compared with the Wt saline group,* $^{b}p < 0.05$ *compared with the transgenic (Tg) saline group.* $^{c}p < 0.05$ *compared with the Wt MPTP group. Scale bars = 500μm (A–D), 1mm (E–H), and 25μm (K).*

uptake of [^{3}H]-MPP$^+$ into striatal synaptosomes (IC_{50}, nM, wild type: 198.00 ± 35.05; Tg: 184.30 ± 20.11; n = 3) and in the accumulation of [^{3}H]-MPP$^+$ in mitochondria (wild type: 100.00 ± 8.29%; Tg: 103.51 ± 3.54 of wild-type control; n = 3). Thus, overexpression of HADH II/ABAD does not confer protection by altering MPTP metabolism or the entry of MPP$^+$ into dopaminergic neurons and subsequently into mitochondria, which are critical steps to the toxicokinetics of MPTP.[27]

HADH II/ABAD Increases Mitochondrial Respiration via Complex I

In mitochondria from wild-type mice, MPP$^+$ inhibited oxygen consumption mediated by complex I in a dose-dependent manner (Fig 4A). However, this inhibition was not as marked in mitochondria from Tg-HADH II/ABAD (see Fig 4A). At a high concentration of MPP$^+$ (100μM), in which approximately 90% of respiration was inhibited, there was no longer any difference in the magnitude of oxygen consumption inhibition between genotypes (see Fig 4A). A similar pattern also was observed when rotenone, a potent lipophilic complex I inhibitor, was used (see Fig 4B), suggesting that the effect of HADH II/ABAD on mitochondrial respiration is not solely restricted to MPP$^+$ but to inhibition of complex I in general. In addition, upon exposure to MPP$^+$, lactate production, an indirect measurement of MPP$^+$-induced complex I inhibition,[28] was lower in striatal tissues of Tg-HADH II/ABAD mice as compared with their wild-type counterparts (see Fig 4F). In well-coupled mitochondria, a higher rate of oxygen consumption correlates with a higher rate of ATP production.[29,30] Consistent with this, a higher rate of oxygen consumption in Tg-HADH II/ABAD mitochondria (see Fig 4A) was indeed associated with higher ATP levels (see Fig 4E).

In contrast to complex I inhibition, no differences between genotypes were detected in the rate of oxygen consumption mediated through complex II (see Fig 4C) and complex IV (see Fig 4D). Thus, these data suggest that upon partial inhibition of complex I, but not of other main electron transport chain enzymatic complexes, HADH II/ABAD can improve mitochondrial respiration and ATP production.

Overexpressing HADH II/ABAD Does Not Alter Mitochondrial Mass or Electron Transport Chain Complex Activities

To explore the mechanism by which HADH II/ABAD improves mitochondrial respiration, we first compared the mitochondrial mass between the two genotypes by measuring the enzymatic activities of citrate synthase, complex I, II, and IV in brain tissue homogenates (Table 2). None of these assays showed any differences between Tg and wild-type mice. Next, we asked whether

Table 1. Levels of Dopamine and Its Metabolites in Striatal Tissues (ng/mg)

	DA	DOPAC	HVA
Wt-saline	13.29 ± 1.41	3.66 ± 0.34	1.15 ± 0.09
Tg-saline	14.12 ± 0.94	3.06 ± 0.20	1.33 ± 0.09
Wt-MPTP	2.77 ± 0.26	1.30 ± 0.15	0.85 ± 0.03
Tg-MPTP	2.83 ± 0.40	2.29 ± 0.12	1.03 ± 0.02

Striatal levels of DA and its metabolites, DOPAC and HVA, were measured using high-performance liquid chromatography. No differences were detected between genotypes. Data represent mean ± SEM of four to five mice per group.

DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; Wt = wild type; Tg = transgenic; MPTP = l-methyl-y-phenyl-1,2,3,6-tetrahydropyridine.

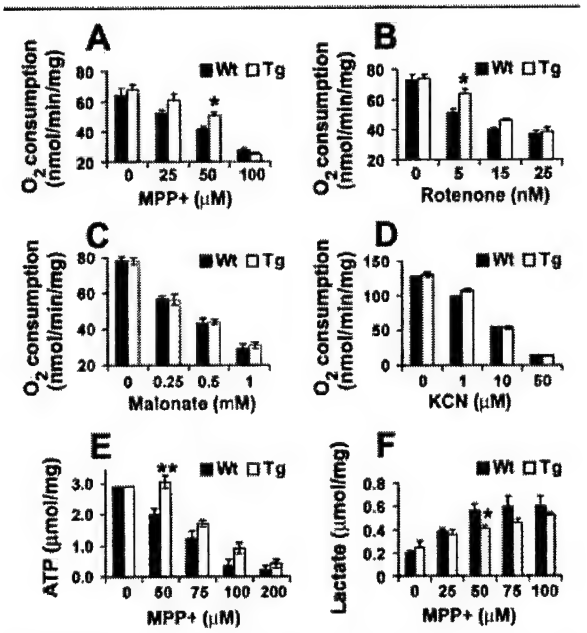

Fig 4. Improvement of mitochondrial functions in HADH II/ABAD mice. Oxygen consumption mediated through complexes I (A, B), II (C), and IV (D) was assessed in the presence or absence of their respective substrates or inhibitors as described in Material and Methods, using purified brain mitochondria. HADH II/ABAD attenuates mitochondrial inhibition specifically via complex I (A, B). Under these identical conditions, HADH II/ABAD also improves ATP production (E). Lactate production in striatal sections confirms MPP^+ has less of an inhibitory effect on mitochondria in transgenic (Tg)–HADH II/ABAD than in their wild-type (Wt) littermates. Data represent mean ± SEM of three to six mice per group, (asterisk) $p < 0.05$ *compared with their respective Wt control groups.*

overexpressing HADH II/ABAD induced permanent structural conformational changes in complex I, thereby reducing the potency of compounds such as MPP^+ or rotenone in blocking its activity. Using purified mitochondria to assess enzymatic activities, we did not detect differences in normal basal activities between Tg and wild-type mice for complexes I, II, or IV or citrate synthase (see Table 2). Because differences in oxygen consumption and ATP production in intact brain mitochondria between genotypes were seen only at certain concentrations of complex I inhibitors, the comparison of complex I enzymatic activity between Tg and wild-type mice was also performed in the presence of different concentrations of MPP^+ (Fig 5A) or rotenone (see Fig 5B). Under this system of opened mitochondria, there were no detectable differences in complex I inhibition at any of the MPP^+ or rotenone concentrations studied between wild-type and Tg-HADH II/ABAD mitochondria (see Fig 5A, B), even when large amounts of HADH II/ABAD recombinant protein were added to the medium (see Fig 5C). Together, these data indicate that, first, the effect of HADH II/ABAD on mitochondrial respiration is not caused by mitochondrial proliferation or complex I catalytic alteration and, second, intact mitochondria are required for HADH II/ABAD to mitigate the impact of complex I inhibition on mitochondrial respiration and ATP production.

Redistribution of HADH II/ABAD from Mitochondrial Matrix to Mitochondrial Membrane

To understand how HADH II/ABAD may sustain mitochondrial respiration under the metabolic stress caused by MPP^+ and rotenone, we assessed the intramitochondrial distribution of this enzyme. In normal respiring brain mitochondria, little HADH II/ABAD is found in the mitochondrial membrane fraction (Fig 6), which is to be expected because this is essentially a mitochondrial matrix enzyme. Conversely, upon incubation of brain mitochondria with MPP^+ or rotenone, there was an increase of HADH II/ABAD content in the mitochondrial membrane fraction. This translocation occurred specifically after complex I inhibition because it was not observed after complex II inhibition produced by malonate (see Fig 6). In contrast, the translocation of another mitochondrial matrix protein, heat shock protein 60 (HSP-60), was not detected (see Fig 6). The present data suggest that, upon complex I inhibition, HADH II/ABAD translocates from the mitochondrial matrix to the inner membrane, thereby allowing this enzyme to be in physical contact or in close

Table 2. Mitochondrial Mass and Activities of Individual Respiratory Chain Complexes (nmol/min/mg protein)

	Brain Homogenates		Brain Mitochondria	
	Wild Type	Tg-HADH II/ABAD	Wild Type	Tg-HADH II/ABAD
Complex I	13.73 ± 1.2	13.27 ± 1	46.5 ± 4.3	42.3 ± 3.6
Complex II	23.37 ± 2.9	21.6 ± 1.5	82.9 ± 1.7	84.0 ± 1.0
Complex IV	888 ± 51.1	951 ± 51.8	3,857.3 ± 67.3	4,145.1 ± 93.0
Citrate synthase	102 ± 13.6	105 ± 3.2	750.49 ± 16.5	796.06 ± 30.0

Brain homogenates and mitochondria were assessed as to whether overexpressing HADH II/ABAD alters the activities of respiratory chain complexes and mitochondrial mass in these animals. No differences were detected between genotypes. Data represent mean ± SEM of three to six mice per group.

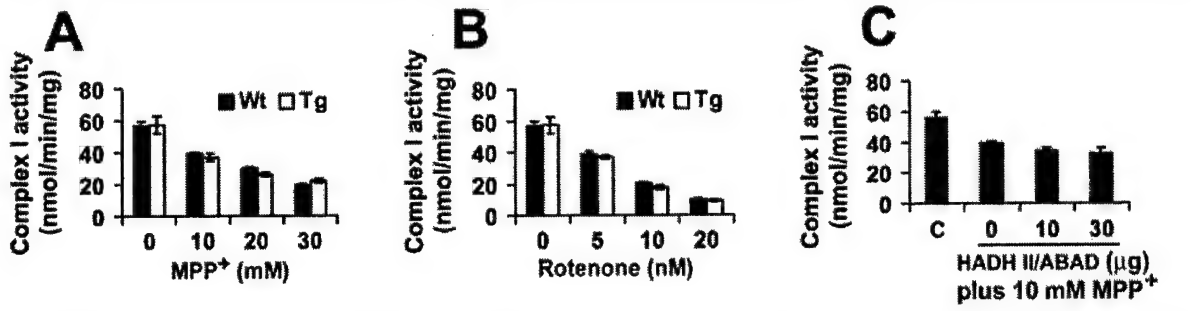

Fig 5. Lack of HADH II/ABAD effects in opened mitochondria. In lysed mitochondria, varying concentrations of MPP^+ (A) and rotenone (B) were used to induce different magnitudes of complex I inhibition like those seen in intact mitochondria in Figure 4. In this system, HADH II/ABAD failed to confer protection, despite the extra supplement of HADH II/ABAD recombinant protein (C). Data represent mean ± SEM of three mice per group.

proximity with complex I (which resides in the inner mitochondrial membrane).

HADH II/ABAD Is Not an Antioxidant

When MPP^+ binds to complex I, in addition to inhibition of mitochondrial respiration and ATP production, it also increases reactive oxygen species (ROS) production. The antioxidant effect of HADH II/ABAD was assessed in purified brain mitochondria obtained from Tg-HADH II/ABAD mice and their wild-type littermates. Table 3 shows that in the presence of MPP^+, HADH II/ABAD did not attenuate ROS production. HADH II/ABAD also did not attenuate ROS production induced by rotenone (data not shown).

Discussion

This study shows that HADH II/ABAD is constitutively expressed in all ventral midbrain neurons, including SNpc dopaminergic neurons, from normal human and mouse tissues. However, HADH II/ABAD tissue content is reduced in affected brain regions from PD patients and MPTP mice. We believe that the reduction of HADH II/ABAD in ventral midbrain in PD and MPTP-treated mice results, at least in part, from a downregulation of HADH II/ABAD and not solely from a loss of HADH II/ABAD-containing neurons for the following reasons. First, in MPTP-intoxicated mice, at the time points when HADH II/ABAD levels in ventral midbrains are reduced, TH levels, used as a phenotypic marker for dopaminergic neurons, are unaffected. The lack of detectable reduction in TH content by Western blot in the face of an approximately 40% reduction in TH-positive neuron numbers is likely caused by SNpc neurons contributing only to a small fraction of the total TH content in the ventral midbrain. Second, as mentioned above, HADH II/ABAD is expressed in all neurons, whereas MPTP only kills dopaminergic neurons. Third, the detection of HADH II/ABAD tissue content is reduced during the active phase of apoptosis, but not when cell loss is stabilized (21 days after MPTP treatment). Although we cannot rule out the possibility of compensatory upregulation of this enzyme at day 21 after MPTP injec-

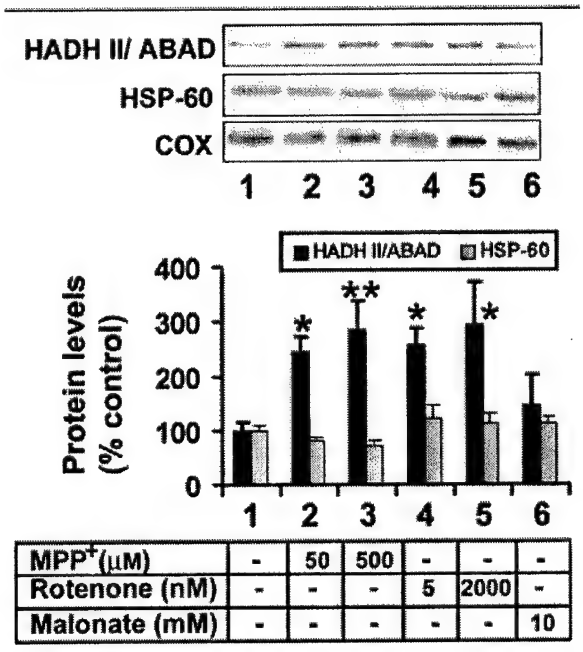

Fig 6. Translocation of HADH II/ABAD. Western blot analyses indicate that under the metabolic stress induced by the complex I inhibitors, MPP^+ or rotenone, HADH II/ABAD but not HSP-60 (another matrix protein) translocated from the matrix to the mitochondrial membrane. Malonate, a complex II inhibitor, failed to induce such an effect. Protein levels were quantified as the ratio of either HADH II/ABAD or HSP-60 over cytochrome c oxidase (COX) and expressed as percentage of their respective controls. Data represent mean ± SEM of six mice per group. (single asterisk) $p < 0.05$; (double asterisks) $p < 0.01$ *as compared with the control group (without inhibitor).*

Table 3. H_2O_2 Production in Purified Brain Mitochondria

Treatment	Mitochondrial H_2O_2 Production (pmol/min/mg protein)	
	Wild Type	Tg-HADH II/ABAD
Control	96.22 ± 12.47	119.09 ± 25.97
MPP^+ (50μM)	94.35 ± 5.0	71.62 ± 6.32
MPP^+ (500μM)	197.53 ± 23.97	160.18 ± 10.60

The fluorescence dye Amplex Red was used to measure H_2O_2 converted from superoxide. HADH II/ABAD does not have an antioxidant effect. Data represent mean ± SEM of three animals per group.

tions, we believe that the lower HADH II/ABAD immunoreactivity in the remaining SNpc dopaminergic neurons in PD tissues argues against this view. The downregulation of HADH II/ABAD in PD and MPTP tissues is not likely a result of a nonspecific alteration in dying neurons because other proteins such as BAX[21] and α-synuclein[31] are increased under these conditions.

Inactivation of the Drosophilae homolog of HADH II/ABAD, termed scully, leads to a lethal phenotype and developmental abnormalities,[32] and in *Caenorhabditis elegans*, reduction in the activities of the short-chain dehydrogenase-reductase family (of which HADH II/ABAD is a member), shortens life span.[33] These findings support a key role for HADH II/ABAD in cell survival and suggest that its downregulation may promote the demise of SNpc dopaminergic cells in PD and in the MPTP mouse model. Consistent with this view is our observation that more SNpc dopaminergic neurons survived in Tg mice overexpressing HADH II/ABAD compared with their wild-type littermates after MPTP administration. When we assessed apoptotic cell death under this MPTP regimen,[21] Tg mice exhibited less apoptotic cells than their wild-type counterparts. We demonstrated that the overexpression HADH II/ABAD was not associated with alterations in one of the key MPTP metabolic steps that determine its potency.[27]

ATP depletion is suspected to be important in MPTP neurotoxicity,[34] and interventions aimed at reducing the cellular energy demand or at increasing the cellular energy stores have all proved to effectively attenuate MPTP-induced neurodegeneration.[9,10,35] These studies highlight the importance of the ATP deficit in the MPTP neurodegenerative process. In normal rodents, dopaminergic structures represent a small fraction of all striatal cellular elements[36] and not much more in the ventral midbrain. This fact renders any reliable detection of ATP changes in brain tissues of MPTP-intoxicated mice[34] precarious. To avoid this problem, we studied the effects of HADH II/ABAD on ATP production and mitochondrial function in purified mitochondria. Through this approach, we demonstrated that mitochondria from Tg-HADH II/ABAD mice had higher oxygen consumption and ATP levels after exposure to MPP^+ compared with mitochondria from their wild-type littermates. These data indicate that overexpression of HADH II/ABAD allows the mitochondria to maintain a higher rate of oxidative phosphorylation and production of ATP in the face of a blockade of complex I. This benefit does not result from permanent structural alterations to the electron transport chain enzymes as evidenced by the lack of difference in complex I enzymatic activity between HADH II/ABAD genotypes. Our data also suggest that, for HADH II/ABAD to confer this protection, intact mitochondria are a prerequisite, perhaps to maintain the intricate interactions between the TCA cycle and the electron transport chain.

Our data further suggest that in active mitochondria, the metabolic stress induced by complex I inhibitors serves as a signal to recruit HADH II/ABAD to the mitochondrial membrane. In contrast, we failed to observe a similar pattern of migration with the mitochondrial matrix marker HSP-60. This latter finding indicates that the increased HADH II/ABAD level in the membrane fraction is not merely a result of nonspecific binding. Once relocated, HADH II/ABAD may increase complex I metabolic efficiency. The failure of HADH II/ABAD recombinant to mitigate the effects of MPP^+ and rotenone on complex I activity in opened mitochondria suggests that the functional interaction of HADH II/ABAD with complex I depends on more than just quantity and proximity.

The exact mechanism by which HADH II/ABAD confers neuroprotection through its suspected interaction with complex I requires further studies. Also, warranting additional investigations is the possible functional link between HADH II/ABAD and Poly(ADP-ribose) polymerase (PARP). This enzyme, which is a DNA binding protein that uses NADH as a substrate, happens to be activated after MPTP administration to mice.[35,37] Thus, HADH II/ABAD overexpression, by generating more NADH, could mitigate the depleting effect of PARP activation on the cellular stores of NADH. Because PARP activation is deleterious in the MPTP model of PD, this scenario may explain, at least in part, how overexpression of HADH II/ABAD confers neuroprotection in the MPTP model of PD.

Blockade of complex I by MPP^+ also stimulates the production of ROS, which have been implicated in inflicting serious oxidative damage to dopaminergic neurons.[27] Here, we have found no evidence that HADH II/ABAD overexpression abates ROS production. The partial nature of the protective effect mediated by HADH II/ABAD is likely due to the fact that while increased expression of this enzyme lessens MPP^+-mediated energy crisis, it does not curtail MPP^+-

mediated oxidative stress. Furthermore, as the rate-limiting enzyme in dopamine synthesis, TH is inactivated by oxidative stress after MPTP injection.[38] This inactivation of TH enzymatic activity may explain the lack of comparable sparing of striatal dopamine and its metabolites against MPTP. Accordingly, in diseases such as PD, where both energy crisis and oxidative stress are presumably instrumental in the neurodegenerative process, optimal neuroprotective strategies for PD may rely on the combination of energy stores-boosting agents and antioxidants.

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Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease

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Degeneration of the nigrostriatal dopaminergic pathway, the hallmark of Parkinson's disease, can be recapitulated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Herein, we demonstrate that adoptive transfer of copolymer-1 immune cells to MPTP recipient mice leads to T cell accumulation within the substantia nigra pars compacta, suppression of microglial activation, and increased local expression of astrocyte-associated glial cell line-derived neurotrophic factor. This immunization strategy resulted in significant protection of nigrostriatal neurons against MPTP-induced neurodegeneration that was abrogated by depletion of donor T cells. Such vaccine treatment strategies may provide benefit for Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disease characterized clinically by resting tremor, rigidity, slowness of voluntary movement, and postural instability (1). Loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), intraneuronal cytoplasmic inclusions or "Lewy bodies," gliosis, and striatal dopamine depletion are principal neuropathological findings. With the exception of inherited cases linked to specific gene defects that account for <10% of cases, PD is a sporadic condition of unknown cause (2).

Inflammation increases the risk of PD (3). Experimental disease models show that innate immunity, especially glial inflammatory factors such as proinflammatory cytokines and reactive oxygen and nitrogen species contribute to the degeneration of the nigrostriatal dopaminergic pathway (4). Although less studied than innate immunity, T lymphocytes present in brain tissue may also affect disease progression (5, 6). For example, T cells perform surveillance functions in the nervous system (7, 8), and T cell-deficient mice show enhanced neuronal loss after CNS damage (9, 10). Adaptive immunity, after vaccination with CNS antigens expressed at the lesion site, can attenuate neuronal death. For instance, in optic nerve and spinal cord injuries, encephalitic T lymphocytes directed against myelin-associated antigens positively affect neurodegenerative processes (11–14). Such self-antigen-stimulated T cells may retard neuronal injury by producing neurotrophins (15, 16) or by influencing their production by local glial cells (17).

Based on these prior studies, we theorized that immunization strategies could induce T cells to enter inflamed nigrostriatal tissue, attenuate innate glial immunity, and increase local neurotrophic factor production. To investigate this notion, copolymer-1 (Cop-1; Copaxone, glatiramer acetate), a random amino acid polymer that generates nonencephalitic T cells, which cross-react with myelin basic protein (MBP) in humans (18) and mice (19), was tested in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Cop-1 immunization protects against secondary CNS injury without the encephalitis associated with MBP immunization (20, 21). Moreover, s.c. Cop-1 immunization preferentially incites T cells with a T_H2 phenotype, which secrete antiinflammatory cytokines such as IL-4, IL-10, and transforming growth factor-β (22). We now demonstrate that Cop-1 immune cells administered to MPTP-intoxicated mice by adoptive transfer enter inflamed brain regions, suppress microglial responses, and increase expression of glial cell line-derived neurotrophic factor (GDNF).‡‡ The process was T cell-dependent and led to significant dopaminergic neuronal protection. Because no currently clinically approved therapy prevents progressive degeneration of dopaminergic neurons in PD, we suggest that such a vaccination strategy could be of therapeutic benefit.

Materials and Methods

Animals and MPTP Treatment. Male SJL mice (6–10 weeks old, The Jackson Laboratory) received four i.p. injections at 2-h intervals of either vehicle (PBS, 10 ml/kg) or MPTP-HCl (18 mg/kg of free base in PBS; Sigma). Twelve hours after the last MPTP injection, random mice received adoptive transfers of splenocytes from Cop-1- or ovalbumin (OVA)-immunized mice or no splenocytes (n = 5–9 mice per group per time point). On days 2 and 7 after MPTP intoxication, mice were killed and brains were processed for subsequent analyses. All animal procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. MPTP handling and safety measures were in accordance with published guidelines (23).

Immunization and Adoptive Transfers. Mice were immunized with a total dose of 200 μg of either Cop-1 or OVA emulsified in complete Freund's adjuvant containing 1 mg/ml *Mycobacterium tuberculosis* (Sigma). Five days after immunization, mice were killed and single-cell suspensions were prepared from the draining inguinal lymph nodes and spleen. MPTP-intoxicated mice received an i.v. injection of 5×10^7 splenocytes in 0.25 ml of Hanks' balanced salt solution. In all adoptive transfer experiments, pooled immunized donor cells were tested for proliferation by [^{3}H]thymidine uptake and/or cytokine expression by ELISA after exposure to immunizing or nonrelevant antigen.

Cytokine Measurements. Donor splenocytes were plated at a density of 1×10^6 cells per ml of tissue culture media [RPMI medium 1640 supplemented with 10% FBS/2 mM L-glutamine/25 mM Hepes/1 mM sodium pyruvate/1× nonessential amino acids/55 μM 2-mercaptoethanol/100 units/ml penicillin/100 μg/ml streptomycin (Mediatech, Herndon, VA)] and stimulated with immunizing antigens. After incubation (37°C at 48 h), supernatants were assayed for IL-10 by ELISA (R&D Systems).

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Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; Cop-1, copolymer-1; MBP, myelin basic protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GDNF, glial cell line-derived neurotrophic factor; OVA, ovalbumin; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein.

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CD90 T Cell Depletion and Flow Cytometry. Donor splenocyte cell suspensions from Cop-1-immunized donors were depleted of T cells using anti-CD90 magnetic beads and magnetic LD columns (Miltenyi Biotec, Auburn, CA). Negatively selected cells ($CD90^-$) were pooled ahead of time and were analyzed for cell purity with a FACSCalibur flow cytometer interfaced with CELLQUEST software (BD Biosciences, Immunocytometry Systems, San Jose, CA) before adoptive transfers. Unfractionated and T cell-depleted populations were stained for T cells using FITC-conjugated anti-CD3 (clone 145-2C11, BD Biosciences, Pharmingen, San Diego) and B cells with phycoerythrin-conjugated anti-B220 (clone RA3-6B2, BD Biosciences, Pharmingen).

Immunohistochemistry and Quantitative Morphology. Seven days after MPTP intoxication, mice were killed and their brains were processed for tyrosine hydroxylase (TH) and thionin staining (24). Total numbers of TH- and Nissl-stained neurons in SNpc were counted stereologically with STEREO INVESTIGATOR software (MicroBrightfield, Williston, VT) by using an optical fractionator (25). Quantitation of striatal TH immunostaining was performed as described (24). Optical density measurements were obtained by digital image analysis (Scion, Frederick, MD). Striatal TH optical density reflected dopaminergic fiber innervation.

Additional primary antibodies used in these studies included rat Mac-1 (1:1,000; Serotec), rabbit glial fibrillary acidic protein (GFAP; 1:1000, DAKO), and rat CD3 (1:800; Pharmingen). Immunostaining was visualized by using diaminobenzidine as the chromogen. For immunofluorescence staining on fresh frozen sections, rabbit anti-CD3 (1:200, DAKO) was used with rat-anti-Mac-1 and goat anti-GDNF (1:100, R & D Systems). Confocal images were obtained with a Zeiss confocal LSM410 microscope.

Cell Tracking. Splenocytes from Cop-1-immunized donors were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) by using the Vybrant CFDA SE cell tracer kit (Molecular Probes). Splenocytes (5×10^7) were adoptively transferred into PBS- or MPTP-treated mice. At 2, 8, and 20 h ($n = 3$ mice per time point) after adoptive transfers, mice were killed, their brains were fixed (4% paraformadehyde), and cryostat-cut sections were analyzed by fluorescence microscopy.

RNA Isolation and Real-Time RT-PCR. Total RNA from ventral midbrain and cerebellum ($n = 5$–7 mice per group) was extracted with TRIzol (Invitrogen). RNA was reverse-transcribed with random hexamers and real-time quantitative PCR was performed on cDNA by using the Applied Biosystems PRISM 7000 sequence detector with SYBR green I as the detection system. The murine primer sequences included: Mac-1, 5′-GCCAATGCAACAGGTGCATAT-3′ (forward) and 5′-CACACATCGGTGGCTGGTAG-3′ (reverse); GDNF, 5′-TGTTCTGCCTGGGTGTTGCT-3′ (forward) and 5′-TTGGAGTCACTGGTCAGCG-3′ (reverse). Primers for GAPDH were purchased from Applied Biosystems. Data are presented as a ratio of mean threshold (C_t) target gene expression and GAPDH. Differences between means were analyzed by using one-way ANOVA followed by the least significant difference posthoc test for pairwise comparisons.

Mac-1$^+$ Immunohistochemistry. Midbrain sections (30 μm) from two mice per treatment group (four to six sections per animal) were immunostained for Mac-1. Cell counts were obtained of amoeboid Mac-1$^+$ cells within the SN by using criteria reported (26) and cells per mm^2 was calculated. Numbers of Mac-1-positive cells were averaged for each animal and the mean cells per mm^2 per animal was estimated. The average countable area between treatment groups ranged from 1.92 mm^2 to 2.22 mm^3, and no significant differences in the size of countable areas were observed by ANOVA ($P = 0.063$, $n = 84$ countable areas).

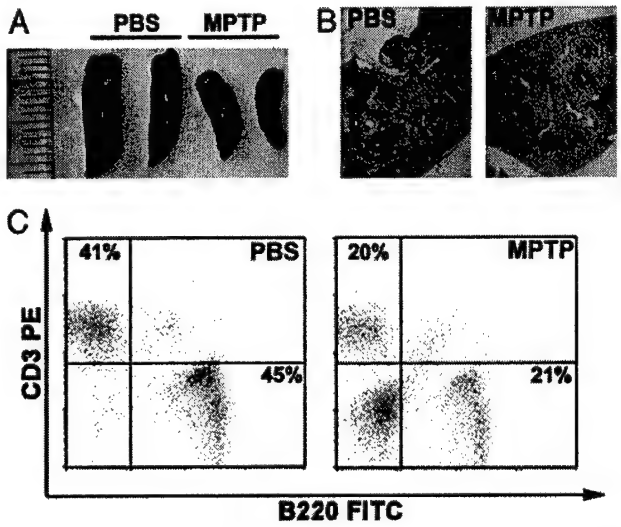

Fig. 1. MPTP-induced immunotoxicity. (*A* and *B*) Seven days after MPTP intoxication, spleen size (*A*) and $CD3^+$ T lymphocyte numbers (*B*) were reduced in spleens of MPTP-treated mice. (*C*) Flow cytometric analysis of splenocytes from PBS (*Left*) and MPTP (*Right*) 2 days after intoxication.

Western Blot Assays. Ventral midbrain protein extracts (25 μg per lane) were fractionated on SDS/4–20% PAGE (Invitrogen), and were then transferred onto PVDF membranes. Membranes were probed with horseradish peroxidase-conjugated anti-mouse IgG or rabbit anti-GFAP (1:15,000; DAKO). Secondary anti-rabbit antibodies conjugated with horseradish peroxidase were visualized by using SuperSignal West Pico chemiluminescent substrate and CCL-XPosure film (Pierce). Immunoblots were stripped and reprobed with antibodies to α-actin (Chemicon) as an internal control.

Measurement of Striatal Catecholamines. Striatal dopamine and its metabolites, dihydroxyphenylacetic acid, and homovanillic acid, were analyzed 7 days after MPTP treatment by reverse-phase HPLC with electrochemical detection (25).

Statistical Analysis. All values are expressed as mean ± SEM. Differences among means were analyzed by one-way ANOVA followed by Bonferroni post hoc testing for pairwise comparison unless otherwise stated. The null hypothesis was rejected at the level of 0.05.

Results

Cop-1 Immunity Confers Dopaminergic Neuroprotection. To test whether Cop-1 immunity confers dopaminergic neuroprotection, MPTP-intoxicated SJL mice received, by adoptive transfer, 12 h after MPTP treatment, 5×10^7 donor splenocytes from nonintoxicated mice previously immunized with either Cop-1 or chicken egg OVA. Replicate MPTP- and PBS-treated mice that did not receive splenocytes served as controls. Adoptive transfer of Cop-1 immune cells to MPTP-treated recipients was used because immunotoxicity precluded active immunization studies. Indeed, MPTP induced significant changes in spleen size with diminished numbers of $CD3^+$ T cells 7 days after MPTP intoxication (Fig. 1 *A* and *B*). Flow cytometric analysis of splenocyte populations revealed a 51% and 53% decrease in $CD3^+$ T cell and $B220^+$ B cell numbers, respectively (Fig. 1*C*). Because MPTP intoxication occurs rapidly and its metabolism into the active toxin, 1-methyl-4-phenylpyridinium (MPP^+), is complete within minutes (27) and is undetectable after 8 h (28), the timing of splenocyte adoptive transfers was designed to avoid confounding effects of MPTP metabolism and its induced hematopoietic toxicity (29). Seven days after MPTP treatment, after which no further dopaminergic neurodegeneration is detected

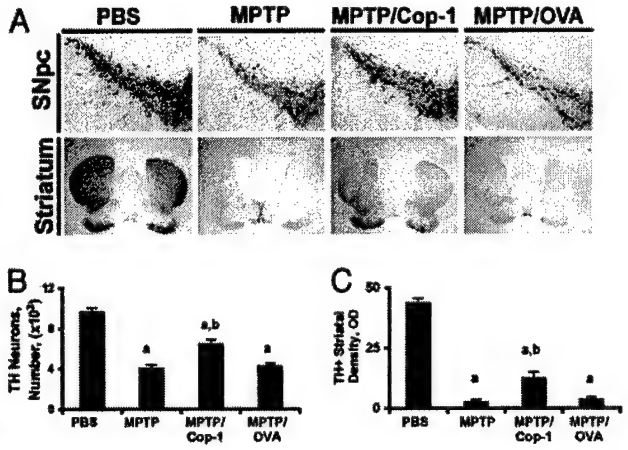

Fig. 2. Cop-1 immunization protects against MPTP-induced dopaminergic neuronal loss. (*A*) Photomicrographs of SNpc and striatum TH immunostaining from PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. (*B*) SNpc TH^+ neuronal counts of SNpc TH^+ neurons. (*C*) Optical densities of striatal TH^+ fibers. Values represent means ± SEM for five to nine mice per group. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b).

(30), mice were transcardially perfused with saline followed by 4% paraformaldehyde, their brains were removed, were cryosectioned, and immunostained for expression of TH, the rate-limiting enzyme in dopamine synthesis (Fig. 2*A*). Stereological counts revealed that MPTP caused a 58% loss of SNpc TH-positive neurons compared with PBS controls (Fig. 2*B*). Similar results were observed in MPTP-injected mice that received splenocytes from OVA-immune donors (MPTP/OVA; Fig. 2 *A* and *B*). In contrast, MPTP-injected mice that received Cop-1 splenocytes (MPTP/Cop-1) exhibited a much smaller reduction in the number of SNpc dopaminergic neurons compared with MPTP or MPTP/OVA animals (Fig. 2 *A* and *B*). Counts of SNpc neurons after Nissl staining with thionin correlated with TH-positive neuron counts ($r = 0.993, P < 0.0001$). This finding confirmed that differences in TH-positive neuron counts were due to numbers of structurally intact neurons and eliminated the possibility that differences resulted from the down regulation of TH itself (Table 2, which is published as supporting information on the PNAS web site, and ref. 30).

Sparing of SNpc dopaminergic cell bodies does not always correlate with protection of their corresponding striatal nerve fibers (25), which is essential for maintaining dopaminergic neurotransmission. To determine whether adoptive transfer of Cop-1 splenocytes affected the integrity of striatal dopaminergic fibers, the density of TH-immunoreactivity in striata (Fig. 2 *A* and *C*) was assessed. MPTP reduced striatal TH density by 94% (MPTP) and 92% (MPTP/OVA) compared with PBS controls (Fig. 2*C*). In contrast, loss of striatal TH density in MPTP/Cop-1 mice (72% loss) was significantly less compared with what was observed in MPTP and MPTP/OVA animals (Fig. 2*C*). The dopaminergic nerve terminals are consistently more affected than the cell bodies in both PD and its MPTP model and are often less amenable to neuroprotection (25, 31). Thus, given the severity of damage at level of the nerve terminals, any significant protection is deemed relevant. Taken together, these findings indicate that Cop-1 immune cells mitigate the deleterious action of MPTP on dopaminergic nerve fibers in the striatum and cell bodies in the SNpc. The ability of splenocytes from Cop-1-immunized mice to confer neuroprotection to myelinated axons is consistent with prior studies where Cop-1 immunization protected against traumatic nerve injury (20).

To determine whether adoptive transfer of Cop-1 immune cells also protects against biochemical deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in striata 7 days after MPTP treatment. Characteristic diminution in striatal dopamine levels by 51% for MPTP-treated mice and 41% for the MPTP/OVA group was observed compared with levels in striata of PBS controls. In contrast, animals that received Cop-1 splenocytes showed only a 4% decrease in striatal dopamine (Table 1). Together, these results indicate that spleen cells from Cop-1-immunized mice protect neuronal dopamine metabolism as well as structural neuronal elements and its projections.

Table 1. Striatal neurotransmitter levels from mice 7 days after MPTP treatment

	Neurotransmitter levels, ng/mg tissue		
Treatment	Dopamine	DOPAC	HVA
PBS ($n = 4$)	10.0 ± 0.1	0.9 ± 0.2	8.8 ± 0.8
MPTP ($n = 6$)	4.9 ± 0.05*	1.4 ± 0.2	5.4 ± 1.1*
COP-1/MPTP ($n = 5$)	9.6 ± 1.1	1.3 ± 0.2	7.7 ± 1.0
OVA/MPTP ($n = 6$)	6.0 ± 0.07*	2.3 ± 0.2†	5.0 ± 0.2*

Values in parentheses are the mean ± SEM for no. of mice per treatment group. DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid.
*$P < 0.01$ compared with PBS and COP-1.
†$P < 0.02$ compared with all groups.

Cop-1 Immune Cells Reduce Microglial Reactions. Based on studies that demonstrate antiinflammatory cytokine profiles by Cop-1-reactive T cells (19, 22), we theorized that the protective effects of Cop-1 immune cells resulted from the modulation of glial inflammatory responses. In line with previously reported results, our immunization strategy generated T cells that proliferate (data not shown) and secrete IL-10 and IL-4 in response to MBP and/or Cop-1 (Fig. 3 *A* and *B*). Because the active phase of neuronal death and neuroinflammatory activities peak at ≈2 days after MPTP injection (25, 30), we assessed lymphocyte infiltration and IgG in the nigrostriatal region at this time point. $CD3^+$ T cells were detected within nigrostriatal tissue (Fig. 3 *C* and *D*) in all mice after MPTP intoxication and adoptive transfer. However, differences were not observed in the ventral midbrain IgG by either Western blot (Fig. 3*E*) or immunohistochemical tests (data not shown). These findings suggested that T cells, not IgG, play the principal roles in the neuroprotective activities observed in these studies. To confirm whether infiltrating T cells in immunized mice were donor-derived, splenocytes were labeled *ex vivo* with the succinimidyl ester of carboxyfluorescein diacetate (Molecular Probes) and transferred intravenously to MPTP mice. As early as 2 h after adoptive transfer and for 20 h thereafter, carboxyfluorescein diacetate-labeled lymphocytes were readily observed both in ventral midbrains and striata of MPTP mice. No labeled cells were found in the cerebellum, a region not afflicted by MPTP. These data demonstrate that donor-derived T cells rapidly enter affected regions of the brain during active inflammation and neuronal loss.

Based on observations that peripheral lymphocytes enter and accumulate in areas of tissue damage early after cell transfer and at times of peak inflammation, we assessed the potential of Cop-1 immune cells to regulate MPTP-induced microglial reactions. In MPTP-treated animals, $CD3^+$ T cells were readily seen in close association with activated microglial cells (Fig. 3*F*); the latter evidenced by increased expression of Mac-1 (CD11b), a cell-surface receptor for complement that is up-regulated by activated microglia in both PD and the MPTP model (31). The evidence that Cop-1 immune spleen cells secreted IL-10 and IL-4 upon *in vitro* stimulation with Cop-1 or MBP (Fig. 3 *A* and *B*), suggested that T cell cytokines may affect glial cell function. Because MPTP-induced neurodegeneration may be attenuated by microglia deactivation (25, 31, 32), we analyzed the ventral midbrain for Mac-1 gene expression by real-time RT-PCR 24 h after adoptive transfer of

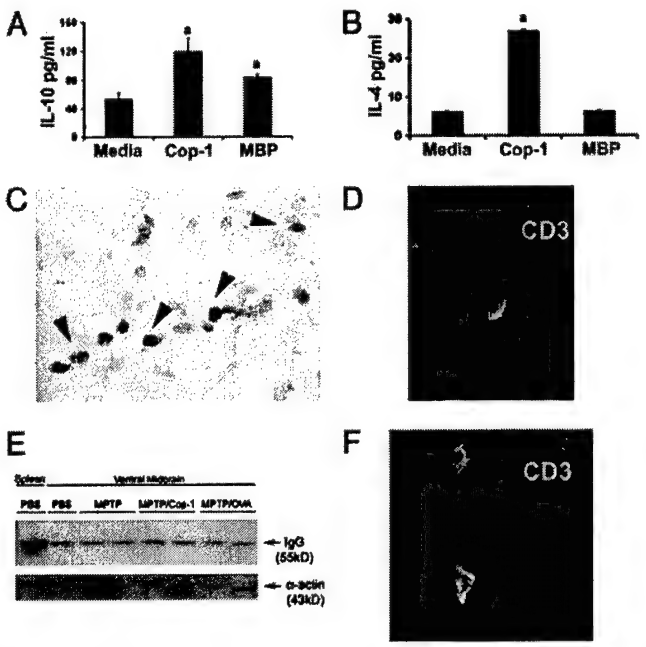

Fig. 3. Cytokine secretion by Cop-1 donor immune cells and T cell infiltration of the SNpc. (*A* and *B*) IL-10 (*A*) and IL-4 (*B*) secretion by Cop-1-immunized splenocytes cultured in media or stimulated with Cop-1 or MBP (30 μg/ml). Values are means of IL-10 or IL-4 concentrations ± SEM for three to four mice. a, $P < 0.05$ compared with PBS treatment group. (*C*) $CD3^+$ T cells in the SNpc of MPTP-intoxicated mice 2 days after adoptive transfer of Cop-1 splenocytes (arrows). (*D*) $CD3^+$ T cells in proximity (green) to TH^+ neurons (red) within the SNpc of an MPTP mouse. (*E*) Western blot analysis for IgG in ventral midbrains after adoptive transfer of splenocytes. (*F*) Ventral midbrain $CD3^+$ T cells (green) in direct contact with Mac-1^+ cells (red; magnification: ×2,000).

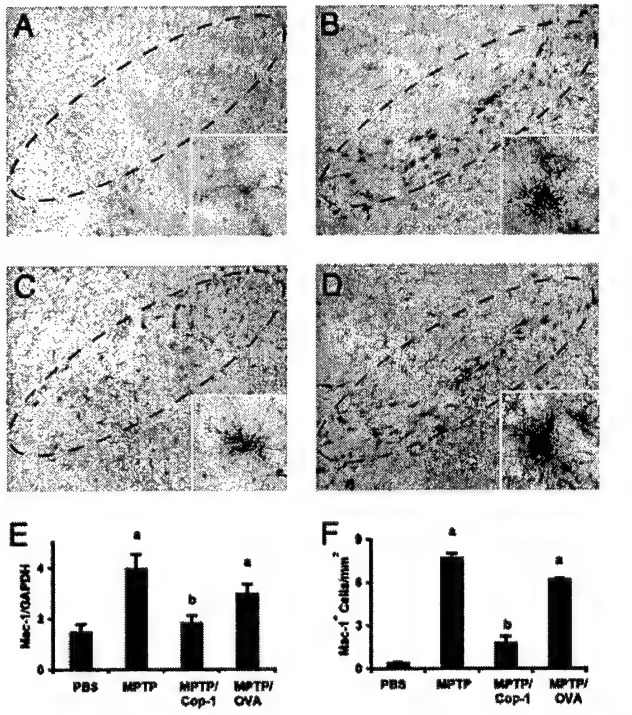

Fig. 4. Cop-1 immunization reduces MPTP-induced microglial reaction in the SNpc. (*A–D*) Mac-1 immunostaining within the SNpc (area circumscribed by dashed line and *Insets* at ×100 magnification) from PBS (*A*), MPTP (*B*), MPTP/Cop-1 (*C*), or MPTP/OVA (*D*) groups. (*E*) Real-time RT-PCR assessment of Mac-1/GAPDH mRNA from ventral midbrain. (*F*) Counts of Mac-1^+-reactive microglia from SNpc. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b) groups.

Cop-1 splenocytes (48 h after last MPTP injection). In agreement with prior studies (31), brains from MPTP-treated animals showed significant increases in Mac-1 mRNA. In contrast, MPTP/Cop-1 mice showed lower Mac-1 expression compared with both MPTP and MPTP/OVA animal groups (Fig. 4*E*). Immunohistochemical staining for cell-surface expression of Mac-1 in the ventral midbrain 48 h after adoptive transfer reflects levels of Mac-1 mRNA (Fig. 4 *A–D*). In PBS control mice, Mac-1 expression was associated with small microglial cells having thin ramifications (Fig. 4*A*). MPTP-injected and MPTP/OVA mice showed intense Mac-1 immunoreactivity, which revealed larger microglial cells with thicker short ramifications (Fig. 4 *B* and *D*). In MPTP/Cop-1 mice, Mac-1^+ cells were smaller, with finer processes approximating those in PBS controls (Fig. 4*C*). Enumeration within the SN of Mac-1^+ microglia with an activated phenotype showed a significant reduction in reactive microglia in the MPTP/Cop-1 group compared with MPTP- or MPTP/OVA-treated mice (Fig. 4*F*). Correlation analysis of Mac-1 mRNA expression and Mac-1^+ microglia counts from PBS-, MPTP-, MPTP/Cop-1-, and MPTP/OVA-treated groups indicated a strong correlation ($r = 0.76, P = 0.03$). Taken together, these data indicate that Cop-1 splenocytes are capable of attenuating MPTP-induced microglial reactions.

Although Cop-1 immune transfer significantly diminished the microglial reaction, astrocyte morphology was not affected. Expression of the astrocyte-specific antigen, GFAP, was comparable among all MPTP treatment groups as revealed by Western blot analysis of ventral midbrain 2 days after MPTP administration (data not shown). Astrocytosis by day 7 after MPTP treatment, shown by enhanced GFAP immunostaining and astrocyte morphology was similar among MPTP-treated groups, irrespective of passive immunization strategies (data not shown).

Neuroprotection Is T Cell-Dependent. As stated, T cells entered the damaged nigrostriatal tissue after MPTP intoxication in the absence of any noticeable alterations in nigrostriatal IgG levels. This finding suggested that the cellular arm of the immune system was responsible for neuroprotection. To test this hypothesis, T cell-depleted splenocytes from Cop-1-immunized mice were prepared by anti-CD90-conjugated magnetic beads. This action resulted in the removal of >90% of $CD3^+$ T lymphocytes without affecting B cell ($B220^+$) populations (Fig. 5*A*). In the experiments, MPTP-treated mice received unfractionated or T cell-depleted splenocytes from Cop-1-immunized donors, unfractionated splenocytes from OVA-immunized donors, or no splenocytes. On day 7, mice were killed and brain tissue was immunostained for TH content in the SNpc and striatum (Fig. 7, which is published as supporting information on the PNAS web site). A significant reduction in the number of TH-positive neurons within the SNpc was observed in MPTP-treated mice that received no splenocytes or splenocytes from OVA-immunized donors (Fig. 5*B*). Significant neuroprotection was afforded to MPTP-treated recipients of splenocytes from Cop-1-immunized mice (Fig. 5*B*). However, neuroprotection was ablated in mice that received T cell-depleted Cop-1 splenocytes (Fig. 5*B*). Parallel changes in striatal dopaminergic nerve fibers was also demonstrated. The diminution of TH optical density in striatal sections was significantly less in MPTP-treated recipients of unfractionated Cop-1 splenocytes compared with MPTP-treated control groups; however, this neuroprotection was ablated in recipients of T cell-depleted Cop-1 splenocytes (Fig. 5*C*). These results indicate that T cells from Cop-1-immunized donors are required for the observed neuroprotective activities.

Cop-1 Immunization Increases Expression of GDNF in Ventral Midbrain. Finally, we investigated whether Cop-1 immunization affects neurotrophin production at the site of disease. GDNF mitigates neu-

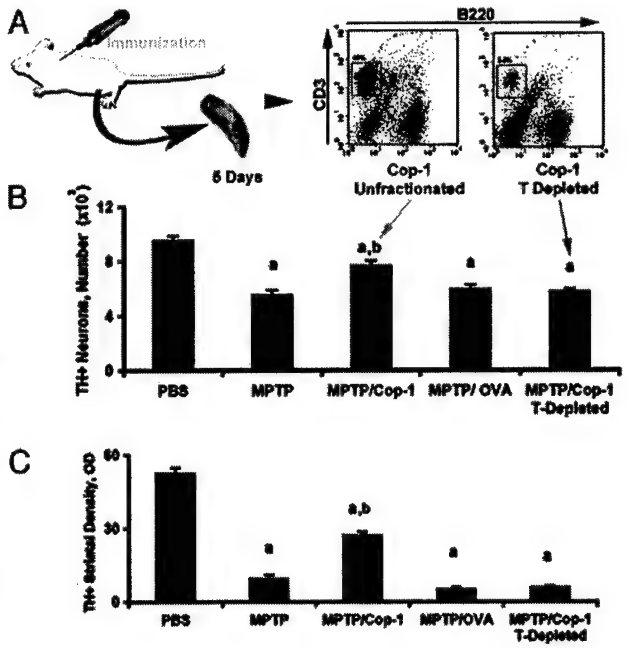

Fig. 5. T cell depletion ablates Cop-1-mediated dopaminergic neuroprotection. (*A*) Flow cytometric analysis of Cop-1 immune splenocytes before (unfractionated) and after T cell depletion. (*B*) Counts of SNpc TH^+ neurons for PBS (n = 5), MPTP (n = 7), MPTP/Cop-1 (n = 8), MPTP/OVA (n = 8), and MPTP/Cop-1/T cell-depleted groups (n = 6). (*C*) Densities of striatal TH^+ fibers. Values are means ± SEM. $P < 0.01$ compared with PBS (a), MPTP (b), and MPTP/OVA (b), MPTP/Cop-1/T (b)-depleted groups.

rodegenerative processes in MPTP animals and leads to symptomatic recovery after dopaminergic injury (33), This finding formed the basis for PD clinical trials that so far have yielded promising results (34). In our study, we quantitated by real-time RT-PCR analysis, GDNF mRNA levels in ventral midbrains from PBS, MPTP, MPTP/Cop-1, and MPTP/OVA mice 20 h after adoptive transfer. MPTP/Cop-1 mice showed significantly greater levels of ventral midbrain GDNF mRNA compared with all other groups (Fig. 6*A*). To identify the cellular source of GDNF within the SN

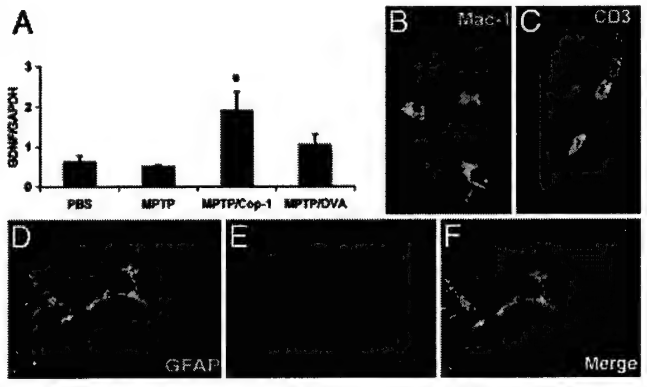

Fig. 6. GDNF expression in MPTP-intoxicated mice after adoptive transfer of Cop-1 splenocytes. (*A*) Real-time RT-PCR of GDNF mRNA expression from ventral midbrains of PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. Values represent ratios of GDNF mRNA normalized to GAPDH and are means ± SEM for five to six mice per group. $P < 0.05$ compared with PBS (a), MPTP, and MPTP/OVA groups. (*B–F*) Confocal microscopy of SNpc from MPTP-treated recipients of splenocytes from Cop-1-immunized mice showing GDNF immunostaining (red) (*B–D* and *F*) and $Mac\text{-}1^+$ (green) microglia (*B*), $CD3^+$ (green) T cells (*C*), and $GFAP^+$ (green) astrocytes (*D* and *F*). Magnification: ×2000.

of MPTP/Cop-1 mice, sections were double immunostained for GDNF and cell markers. Analysis by confocal microscopy demonstrated that GDNF expression colocalized with cells expressing GFAP, but not with CD3 or Mac-1 (Fig. 6 *B–F*). These data suggest that astrocytes, not T cells or microglia, are the primary source of GDNF production in this model.

Discussion

Epidemiological, immunopathological, and animal model studies support the notion that innate immunity affects nigrostriatal dopaminergic neurodegeneration in PD (2, 35). Many of the pathogenic processes operative in PD are recapitulated in MPTP-intoxicated animals. For example, animals injected with MPTP exhibit early microglial-associated neuroinflammatory events and subsequent nigrostriatal degeneration. Based on a number of prior studies linking neuroinflammation to neurodegenerative processes, we hypothesized that negatively regulating innate immunity in the CNS through T_H2-polarized adaptive immune responses through vaccination could lead to positive disease outcomes. Consistent with this idea, we demonstrate that passive immunization with Cop-1 immune cells into acutely MPTP-intoxicated mice protects the nigrostriatal dopaminergic system. This finding was evidenced by higher numbers of surviving SNpc TH^+ neuronal bodies and striatal fibers, in addition to elevated striatal dopamine levels in MPTP mice receiving Cop-1 immune cells. Taken together, the data indicate that Cop-1 immune cells accumulate specifically in affected brain areas during the most active phase of MPTP-induced neurodegeneration (30), and by so doing, trigger a T cell-dependent neuroprotective response.

The neuroprotection seen in our studies could result as a consequence of T_H1 (proinflammatory, IFN-γ) or a T_H2 or T_H3 (antiinflammatory, IL-10, IL-4, and TGF-β) immune response. However, Cop-1 immunization, in particular, is well known to generate T_H2 or T_H3 T cells (19, 22), which secrete cytokines known to suppress innate immunity (36–38). Cop-1 immunization, in the MPTP model, could exploit immunoregulatory activities of T_H2 or T_H3 T cells and thus provide a vehicle to attenuate microglial neurotoxic responses. Several of our observations support the notion that this scenario may underlie, at least in part, Cop-1 neuroprotective effects in the MPTP model. First, infiltration of the nigrostriatal pathway with donor-derived T cells was seen in close proximity to or in direct cell–cell contact with activated microglia. Second, a marked decrease in MPTP-associated microglial responses was observed after transfer of Cop-1 immune cells. This finding was supported by a profound reduction of ventral midbrain Mac-1 mRNA content and SNpc Mac-1 immunostaining. Third, MPTP-associated astrocytosis, a putative neuroprotective response, remained unchanged by passive immunization with Cop-1 cells. Fourth, IL-10 and IL-4, but not IFN-γ, was secreted by the Cop-1 cells in laboratory assays, providing evidence for the induction of an antiinflammatory T_H2 phenotype in affected brain tissue. Taken together, these results suggest that Cop-1 immune cells in MPTP mice can attenuate the microglial inflammatory responses that contribute to nigrostriatal dopaminergic neurodegeneration.

In addition to targeting the innate immune system, this therapeutic vaccine strategy was shown to augment GDNF within brain regions of active disease. It is likely that this effect is also implicated in the neuroprotective activities of Cop-1 because GDNF delivered to MPTP-intoxicated animals shows significant benefit (33). This observation may also be relevant to human disease given the therapeutic benefits of surgically implanted pumps which directly infuse GDNF into affected dopaminergic structures of PD patients in early human clinical trials (34). Activated T cells express both neurotrophins (39) and the neurotrophic factor receptors, trkB and trkC (40), thus providing sufficient mechanistic means to establish T cell–neuron communications. Consistent with this view, T cells can increase local CNS neurotrophic factor production *in vivo* (11). Our data demonstrate a dramatic increase in ventral midbrain

GDNF expression in Cop-1-immunized MPTP-injected mice. Interestingly, confocal microscopy revealed GDNF in astrocytes, but not in microglia or infiltrating T cells. Thus, these findings suggest that Cop-1 immune cells stimulate the local production of GDNF by astrocytes. In keeping with this idea, T cell cytokines are well known to affect the regulation of neurotrophins (17, 41) that in turn, could actively participate in the observed Cop-1-induced neuroprotective effects.

To our knowledge, this is the first time that a vaccine strategy has been used to confer neuroprotection for dopaminergic neurons. We posit that Cop-1-specific T_H2 cells, which recognize MBP, simultaneously suppress cytotoxic inflammatory responses and increase local neurotrophic factor production. It is possible that both mechanisms converge to ultimately abate the dopaminergic neurodegenerative process that occurs in the MPTP model of PD. In this regard, Cop-1 vaccination reflects the anticipated outcomes of gene therapy. Indeed, both approaches attempt to deliver factors that would attenuate disease to damaged microenvironments. This method is implemented to enhance the therapeutic index by delivering maximal levels of factors to specific diseased areas, thus minimizing system toxicity. Still, immunization avoids the inherent limitation of gene delivery and, by directing immune cells to areas of injury and producing a spectrum of disease mitigating factors, positively alters the neurodegenerative process. Additional studies performed in the MPTP model wherein animals analyzed over time and TH^+ neuronal counts substantiated with other tests, including behavioral and spectroscopic assays, may serve to further validate our experimental observations. In keeping with this concept, preliminary reports from our laboratories based on measures of *N*-acetyl aspartate (a biochemical neuronal marker) by using magnetic resonance spectroscopy and immunopathological coregistration and reverse-phase HPLC in the SNpc confirmed the neuroprotective effects of Cop-1 immune cells in MPTP-injected mice.[§§]

We found unexpectedly that MPTP induces a profound toxicity on cellular components of the peripheral immune system. Adoptive transfer of Cop-1 immune cells to MPTP recipient animals was used as immunotoxicity precluded active immunization. Moreover, our initial work used adoptive transfers with whole splenocyte populations to determine whether collective immune responses elicited against Cop-1 and reflecting active immunization could elicit neuroprotective activities in an animal model of PD. This step is critical in preclinical studies because both T and B cells can affect outcomes in nerve injury models (9) and may work in concert in doing so (42). Indeed, the pharmacokinetics of MPTP are well studied and demonstrate that the toxin is rapidly metabolized in mice and no longer detectable 8 h after the final dose (28). Although passive transfer is commonly performed in humans, there are no contraindications for PD patients to receive direct vaccination with Cop-1 or other related antigens that might elicit similar neuroprotective responses.

We conclude that this report opens a field of investigation toward the development of neuroprotective therapeutic modalities for PD. The reported Cop-1-specific immune-mediated neuroprotection has direct implications for the treatment of PD. As a Food and Drug Administration-approved and well tolerated drug, Cop-1 has been used effectively in patients with chronic neuroinflammatory disease such as relapsing remitting multiple sclerosis for more than a decade. Given the safety record of Cop-1 and that current treatments for PD remain palliative, such a vaccination strategy represents a promising therapeutic avenue that can readily be tested in human clinical trials.

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[§§]Boska, M. B., Mosley, R. L., Lewis, T. B., Nelson, J., Benner, E. J. & Gendelman, H. E. 33rd Annual Meeting of the Society for Neuroscience, Nov. 8–12, 2003, New Orleans, LA, abstr. 440.2.

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MPTP as a Mitochondrial Neurotoxic Model of Parkinson's Disease

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1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin extensively used to model Parkinson's disease (PD). A cascade of deleterous events, in which mitochondria play a pivotal role, drives MPTP neurotoxicity. How mitochondria are affected by MPTP and how their defect contributes to the demise of dopaminergic neurons in this model of PD are discussed in this review.

KEY WORDS: MPTP; Parkinson's disease; neurodegeneration; mitochondria; oxidative stress; ATP depletion; programmed cell death.

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects (Ziering *et al.*, 1947). MPTP can induce a parkinsonian syndrome in humans and nonhuman primates almost indistinguishable from Parkinson's disease (PD) on both clinical and neuropathological standpoints (Langston and Irwin, 1986). Over the years, MPTP has been used in a host of different animal species, especially in mice (Heiklla *et al.*, 1989), to recapitulate the hallmark of PD cellular pathology, namely the degeneration of the nigrostriatal dopaminergic pathway (Dauer and Przedborski, 2003). Although the MPTP model departs from PD on several significant aspects, it continues to be regarded as the best experimental model of this common neurodegenerative disease. With respect to PD, enthusiasm for the MPTP model is driven by the belief that unraveling the MPTP neurotoxic process in animals may provide hints into the mechanisms responsible for the demise of dopaminergic neurons in human PD.

Various key cellular and molecular components underlying the MPTP neurotoxic process have been reviewed in details in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) and will thus not be discussed here. Instead, the focus of this minireview will be devoted to the role of the mitochondria in the deleterious effects of the parkinsonian toxin MPTP.

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FIRST STEP FIRST

MPTP is a protoxin whose toxicokinetics is a complex, multistep process (Dauer and Przedborski, 2003). As indicated by its octanol/water partition coefficient of 15.6 (Riachi *et al.*, 1989), MPTP is a highly lipophilic molecule, which is able to readily permeate lipid bilayer membranes. It is therefore not surprising to observe that MPTP crosses the blood-brain barrier in a matter of seconds after its systemic administration (Markey *et al.*, 1984). Once in the brain, it is rapidly converted into 1-methyl-4-phenylpyridinium (MPP^+), the actual neurotoxin (Heikkila *et al.*, 1984). This critical transformation of MPTP into MPP^+ is a two-step process. First, MPTP undergoes a two-electron oxidation, catalyzed by monoamine oxidase B (MAO-B), yielding the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (Chiba *et al.*, 1984). Given the discrete cellular distribution of MAO-B in the brain (Kitahama *et al.*, 1991), it is believed that the conversion of MPTP to $MPDP^+$ occurs specifically in glial and serotonergic cells, and not in dopaminergic neurons. $MPDP^+$ is an unstable molecule which readily undergoes spontaneous

disproportionation to MPP^+ and MPTP (Chiba *et al.*, 1985; Peterson *et al.*, 1985).

Once formed, MPP^+ is presumably released from glial and serotonergic cells into the extracellular space prior to entering dopaminergic neurons. Yet, MPP^+ has an octanol/water partition coefficient of 0.09 (Riachi *et al.*, 1989), which indicates that, while being a lipophilic cation, MPP^+ is far less lipophilic than MPTP. Thus, unlike MPTP, MPP^+ is most likely unable to easily diffuse across cellular lipid bilayer membranes. Instead, it is to be expected that the release of MPP^+ from its intracellular sites of formation and entry into adjacent neurons depend on specialized carriers. Consistent with this view is the fact that MPP^+ access to dopaminergic neurons relies on the plasma membrane dopamine transporter (Bezard *et al.*, 1999; Javitch *et al.*, 1985).

MITOCHONDRIAL ACCUMULATION

Once inside neurons, MPP^+ rapidly accumulates in the mitochondrial matrix (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). Initially, it was thought that MPP^+ gains access to the mitochondrial matrix through a carrier (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). However, it is now well established that MPP^+ is passively transported (Davey *et al.*, 1992; Hoppel *et al.*, 1987) by a mechanism relying entirely upon the large mitochondrial transmembrane potential gradient ($\Delta\psi$) of -150 to -170 mV (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987; Ramsay *et al.*, 1986; Ramsay and Singer, 1986).

Like with other lipophilic cations (Rottenberg, 1984), the higher the concentrations of intramitochondrial MPP^+, the lower the $\Delta\psi$ and, consequently, the slower the uptake of extramitochondrial MPP^+ (Davey *et al.*, 1992; Hoppel *et al.*, 1987). The demonstration that the ion-pairing agent tetraphenylboron anion increases both the rate and the extent of MPP^+ uptake in isolated mitochondria (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987) further supports this concept. As discussed below, MPP^+ inhibits mitochondrial respiration, which likely also contributes to the loss of the $\Delta\psi$ gradient and to the dampening of the mitochondrial uptake of MPP^+. It is thus not surprising that the accumulation of MPP^+ by energized mitochondria behaves as a saturable phenomenon in the presence of high extramitochondrial concentrations of MPP^+ (e.g., >10 mM) (Ramsay and Singer, 1986) and appears to reach a steady state after a few minutes (Davey *et al.*, 1992; Ramsay *et al.*, 1986). This apparent steady state persists until mitochondrial suspension becomes anaerobic or $\Delta\psi$ is collapsed by the addition of an uncoupler agent such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Ramsay *et al.*, 1986). Remarkably, energized mitochondria incubated with 0.5 mM MPP^+ reach matrix concentrations of more than 24 mM after only 10 min (Ramsay and Singer, 1986). This fast and avid uptake suggests that most, if not all, of the cytosolic MPP^+ would eventually accumulate in the mitochondrial matrix after the systemic injection of MPTP.

INTRAMITOCHONDRIAL MPP^+

It is well established that intramitochondrial MPP^+ inhibits oxidative phosphorylation (Nicklas *et al.*, 1985; Singer *et al.*, 1987). Intramitochondrial MPP^+ also appears to inhibit the tricarboxylic acid cycle enzyme α-ketoglutarate dehydrogenase (Mizuno *et al.*, 1987a). Although both mitochondrial metabolic alterations may contribute to MPP^+ cytotoxicity, attention has been paid almost exclusively to the action of MPP^+ on the respiratory chain.

It is well documented that MPP^+ impairs, in a dose- and time-dependent manner, the ADP-stimulated oxygen consumption (State 3) in intact mitochondria supported by the NADH-linked substrates glutamate and malate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). MPP^+ is, however, ineffective in inhibiting the oxygen consumption in mitochondria supported by succinate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). Furthermore, MPP^+ prevents the binding of the classical Complex I inhibitor [^{14}C]-rotenone to electron transport particles (Ramsay *et al.*, 1991a). Collectively these findings indicate that MPP^+, like rotenone and piericidin A, impairs mitochondrial respiration by inhibiting the multi-subunit enzyme Complex I (i.e., NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain. This straightforward interpretation is supported by the electron spin resonance demonstration that MPP^+ does actually bind to Complex I and blocks the terminal step of electron transfer from the highest potential iron–sulfur cluster of Complex I called N2 to ubiquinone (Ramsay *et al.*, 1987).

The use of several MPP^+ analogs and cationic inhibitors has demonstrated that MPP^+ binds at two distinct sites within the mitochondrial electron transport chain region comprised between N2 and ubiquinone (Gluck *et al.*, 1994; Miyoshi *et al.*, 1997, 1998; Ramsay *et al.*, 1989, 1991b; Ramsay and Singer, 1992). These studies have also demonstrated that the occupation of both sites appears to be required for complete inhibition of NADH oxidation. The binding of MPP^+ to the first, more *hydrophilic* site seems to primarily affect the functional coupling between the PSST and the ND1 subunit of Complex I and to account

for only 40% of the MPP^+-induced reduction in NADH oxidation (Schuler and Casida, 2001). The binding of MPP^+ to the second, more *hydrophobic* site seems quite potent in blocking Complex I enzymatic activity (Schuler and Casida, 2001). Yet, the exact location of this second binding site in Complex I remains to be determined. Nonetheless, the importance of the binding to PSST, but not to the ND1 subunit in the inhibition of Complex I-mediated NADH oxidation (Schuler *et al.*, 1999; Schule and Casida, 2001), suggests that the MPP^+ *hydrophobic* site must also be situated somewhere in the PSST subunit. This *hydrophobic* site appears not to exist for other typical Complex I inhibitors such as rotenone and piericidin A (Schuler and Casida, 2001). Accordingly, while MPP^+ binds to Complex I, as do rotenone and piericidin A (Gluck *et al.*, 1994; Krueger *et al.*, 1993), it may not bind to exactly the same Complex I subunit or subunit part as these two other Complex I inhibitors. Also worth noting is the fact that MPP^+, compared to rotenone and piericidin A, is a far weaker inhibitor of Complex I, which may explain why millimolar concentrations of MPP^+ are needed to inhibit NADH-oxidation in electron transport particles (Hoppel *et al.*, 1987).

CONSEQUENCES OF MPP^+-INDUCED COMPLEX I INHIBITION

In response to MPP^+ binding to Complex I, the flow of electrons along the respiratory chain is hampered in both dose- and time-dependent manners (Hasegawa *et al.*, 1990; Nicklas *et al.*, 1985; Vyas *et al.*, 1986). The importance of the inhibition of Complex I in the MPTP-induced neurotoxicity in vivo is supported by the demonstration that strategies aimed at stimulating oxidative phosphorylation via by-passing the blockade of Complex I not only improve mitochondrial respiration but also mitigate dopaminergic neurodegeneration in mice (Tieu *et al.*, 2003).

The current hypothesis on MPTP cytotoxicity posits that one of the main contributors to cell death is the impaired synthesis of ATP resulting from the inhibition of Complex I by MPP^+. Relevant to this view is the fact that MPP^+ indeed causes a rapid and profound depletion of cellular ATP levels in isolated hepatocytes (Di Monte *et al.*, 1986), in brain synaptosomal preparations (Scotcher *et al.*, 1990), and in whole mouse brain tissues (Chan *et al.*, 1991). It appears, however, that Complex I activity should be reduced by more that 50% to cause significant ATP depletion in nonsynaptic brain mitochondria (Davey and Clark, 1996). Furthermore, in vivo MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (Chan *et al.*, 1991). These facts argue against MPP^+-related ATP deficits being the sole factor underlying MPTP-induced cell death.

Another consequence of Complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS). It was shown that incubation of MPTP with brain mitochondria resulted in an oxygen-dependent formation of ROS (Rossetti *et al.*, 1988). It was also shown that incubation of MPP^+ with bovine heart submitochondrial particles causes a production of superoxide radicals when MPP^+ is used at the concentrations expected to be found inside neurons after MPTP systemic administration (Hasegawa *et al.*, 1990). In this study, the authors also demonstrate that the degree of Complex I inhibition is proportional to the amount of superoxide radical produced (Hasegawa *et al.*, 1990). Because modulations of key mitochondrial ROS scavengers, such as manganese superoxide dismutase, affect MPTP-induced neurotoxicity in mice (Andreassen *et al.*, 2001; Klivenyi *et al.*, 1998), it is reasonable to assert that MPP^+-related ROS production also contributes to MPTP-induced cell death.

CONCLUSION

As discussed above, ATP depletion and ROS overproduction appear to occur soon after MPTP injection, subjecting the intoxicated cells, early on, to an energy crisis and oxidative stress. However, the time course of these perturbations reviewed in the following reference (Przedborski and Vila, 2003) appears to correlate poorly with the time course of neuronal death in vivo (Jackson-Lewis *et al.*, 1995). What this meta-analysis is suggesting is that only a few neurons are probably succumbing to the early combined effects of ATP depletion and ROS overproduction. Instead, mounting evidence discussed in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) indicates that rather than killing the cells, alterations in ATP synthesis and ROS production are pivotal in triggering cell-death-related molecular pathways which, once activated, rapidly lead to the demise of the intoxicated neurons.

Interestingly enough, among these latter molecular pathways, it appears that the mitochondrial-dependent programmed cell death machinery plays a critical role (Vila *et al.*, 2001). As illustrated in Fig. 1, it is thus plausible that the death of neurons caused by MPTP results from a circular cascade of deleterious events starting at the mitochondria by the alteration of the oxidative phosphorylation and finishing also at the mitochondria by the activation of the programmed cell death machinery. Whether the whole circuit depicted above is entirely orchestrated

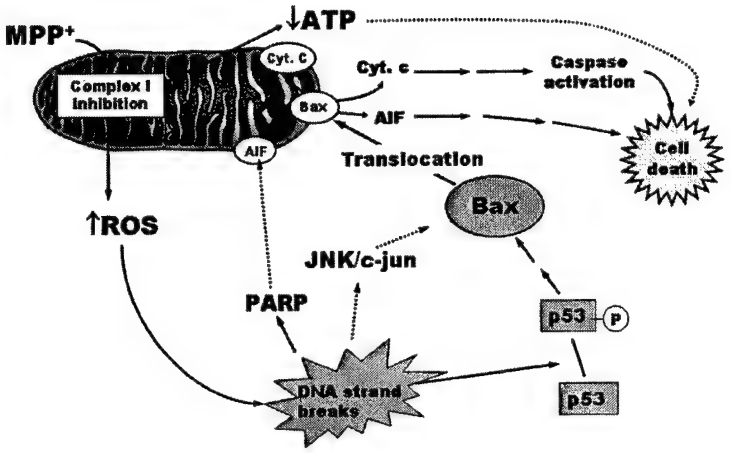

Fig. 1. Illustration *of the proposed circular nature of the MPP$^+$-mediated cell death cascade* MPP$^+$ enters in the mitochondrion and binds to Complex I, whereby it inhibits ATP synthesis and stimulates ROS production. These two initial events lead to a host of cellular perturbations such as DNA damage, which, in turn, trigger a variety of cell-death-related pathways. These include activations of p53 by phosphorylation (p53-*p*) and JNK/c-Jun, which lead to Bax induction and translocation to the mitochondria. DNA damage also stimulates poly(ADP-ribose) polymerase (PARP) activity. Bax translocation and PARP activation promote the translocation of cytochrome *c* and apoptosis-inducing factor (AIF) from the mitochondria to the cytosol. Once in the cytosol, cytochrome c participates in a caspase-dependent cell death process, while AIF participates in a caspase-independent cell death process, both of which are not necessary mutually exclusive. Solid arrow, known mechanism; dashed arrow, speculated mechanism.

at the level of the mitochondria or whether it also involves perturbations that arise in the cytosol (e.g., protein nitration, cyclooxygenase-2 induction) and the nucleus (e.g., DNA damage, PARP activation) of the intoxicated cells is the focus of several ongoing studies in our laboratory.

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CHAPTER

B3

From Man to Mouse: The MPTP Model of Parkinson Disease

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In 1817 Dr. James Parkinson first described the syndrome that we know today as Parkinson disease (PD) in a paper entitled "An Essay on the Shaking Palsy" (Parkinson 1817). PD is a debilitating neurological disorder that strikes approximately 1–2% of the adult population older than fifty years of age (new incidence is 20 per 100,000 persons) (de Rijk et al. 1995). Current estimates from the American Parkinson Disease Foundation put the number of American citizens suffering from this disease at greater than one million persons. The costs of treatment of PD can be staggering. At an average per patient cost of $6,000per year—for drugs, physicians, and loss of pay to patient and family members—(Whetten-Goldstein et al. 1997), the total cost of the disease may approach $6,000,000,000 per year; of which 85% is borne by private and government (e.g., Social Security, Medicare) insurance. In fact, more individuals present with PD than with multiple sclerosis, muscular dystrophy, and amyotropic lateral sclerosis (Lou Gehrig disease) combined (The Parkinsons Web, 1997). Since the population of the world is, on average, getting progressively older (United States Census Bureau, 1996), the number of people suffering from this disease should increase substantially within the next several decades. Furthermore, PD is an incurable disease with an average life expectancy after diagnosis of over fifteen years, thus there should be an even larger burden on both the social and financial resources of families, insurance companies, and the federal government than is present today.

I. BACKGROUND

Parkinson disease is characterized by a loss of the pigmented cells located in the midbrain substantia nigra pars compacta (SNpc). The loss of these cells causes a reduction in afferent fibers that project to the striatum. PD symptoms first manifest when approximately 60% of the SNpc neurons have already died (German et al. 1989). Because the progression of cell loss is thought to occur over a somewhat protracted period of time in a defined spatiotemporal manner (Damier et al. 1999; Nurmi et al. 2001), the onset of Parkinson disease symptoms is often insidious.

The underlying cause for the vast majority of PD cases is unknown. Controversy still exists as to how much of the disease results from a strict genetic causation, a purely environmental factor, or the more parsimonious combination of the two risk factors (Duvoisin 1999; Williams et al. 1999; Gasser 2001). Empirical evidence suggests that less than 10% of all diagnosed Parkinsonism has a strict familial etiology (Payami and Zareparsi 1998). A small number of

familial parkinsonian patients appear to have polymorphisms in the α-synuclein gene (Polymeropoulos et al. 1997), suggesting that this aggregating protein (Spillantini et al. 1997) may play a role in Lewy body formation that ultimately results in substantia nigra cell death (Nussbaum and Polymeropoulos, 1997). A second autosomal recessive locus coding for the parkin protein maps to the long arm of chromosome 6 (6q25.2-q27). Mutations in this gene cause a form of juvenile onset PD. Other genes that are associated with PD include loci at human chromosome 2p13 and 4p (Gasser, 2001). The PD linked to this locus more closely resembles that of idiopathic PD, although like the α-synuclein protein, this unknown protein has very low penetrance. However, at this time no mutations in these proteins are reported in idiopathic PD (Hu et al. 1999; Scott et al. 1999).

Because the vast majority of PD patients have no direct tie to any identified genetic mutation, important information regarding the pathophysiology of PD may be gleaned through the study of animal models. Several animal models examined the mechanism(s) underlying the pathophysiology of experimental PD, including surgical and chemical models. One of the earliest models made use of a lesion of nigrostriatal pathway in which fibers emanating from the substantia nigra proceeded to the striatum rostrally through the medial forebrain bundle (Faull and Mehler 1978; Levine et al. 1983; Brecknell et al. 1995). In addition to the physical lesion studies, chemical lesions also modeled Parkinson disease. In these studies, animals were injected with 6-OHDA, a neurotoxin that when injected into the striatum causes a retrograde degeneration of dopaminergic neurons in the SNpc (reviewed in Olney et al. 1990; Schwarting and Huston 1996; Deumens et al. 2002; Hirsch et al. 2003). A third model of experimental PD utilizes the properties of selective neurotoxins, the most famous of which is the loss of SNpc neurons following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

II. MPTP

The discovery of MPTP has provided a useful model of Parkinsonism that appears to recapitulate the pathology of the disease seen in humans. The identification of MPTP may be one of the few cases in which a specific neurotoxin was discovered in humans first, followed by development of an animal model. The story first started around 1976 when a chemistry student named Barry Kidston was synthesizing a "designer" heroin, MPPP, for recreational use. Although generally successful, at one point he hurried the catalysis of the procedure and instead of producing MPPP, he synthesized a neurotoxin that a team from the National Institutes of Mental Health later found to be MPTP. After IV injection of the incorrectly designed drug, Kidston quickly exhibited a severe bradykinesia. Following a rapid hospitalization and initial diagnosis as a catatonic schizophrenic, physicians eventually suspected that he had an acute form of Parkinson disease. Kidston's symptomatic recovery after he was administered L-dopa confirmed suspicions. Because this was an isolated case, the details never attained public prominence, but this changed in the early 1980s after a number of northern California heroin users were identified who presented at various emergency rooms with symptoms indistinguishable from those of Parkinson disease (Burns et al. 1985; Langston 1985). The potential threat of a public health risk that could have been epidemic, brought the case of these "frozen addicts" to public awareness (Langston 1985). A complete history of these cases is presented in the book *The Case of the Frozen Addicts* (Langston and Palfreman 1996) as well as in the NOVA documentary of the same name (original broadcast date: February 18, 1986).

In the subsequent years since MPTP was identified in humans as a Parkinsonian agent, researchers have demonstrated that MPTP exerts its neurotoxic effects in a number of other primates (Kopin and Markey 1988; Jenner 2003; Wichmann and DeLong 2003), as well as in cats, and in several rodents. In rodents, only specific strains of mice are sensitive to the administration of MPTP (Sundstrom et al. 1987; Riachi and Harik 1988; Mitra et al. 1994; Hamre et al. 1999). MPTP structurally resembles several known environmental agents, including well- known herbicides such as paraquat (Di Monte et al. 1986) and garden insecticides and fish toxins such as rotenone (McNaught et al. 1996) that induce dopamine cell degeneration (Brooks et al. 1999; Betarbet et al. 2000; Thiruchelvam et al. 2000; Chun et al. 2001). As such, it is possible, although as of yet unproven, that the genetic pathways and mechanisms that underlie the toxin-induced cell death of each of these compounds may interact.

There are many points systemically where MPTP can affect the dopaminergic system (fig. 1). In this chapter, we will discuss each step in the MPTP toxification pathway.

Step 1. Introduction of MPTP into the CNS

MPTP, in and of itself, is not toxic. The enzyme MAO-B metabolizes MPTP to the unstable 1-methyl-4-phenyl-2, 3-dihydropyridium ($MPDP^+$) that then rehydrogenates or deprotonates to generate MPTP or the corresponding pyridium species, MPP^+, respectively (fig. 2). At the point of interface with the periphery, exogenous compounds can either enter or be excluded from the CNS by the blood-brain-barrier (BBB). The BBB is composed on tight-junctioned endothelial cells that make up the microvasculature of the brain in tight opposition with the end feet of glial processes. Endothelial cells of the microvasculature contain monoamine oxidases, and several studies have correlated levels of monoamine oxidases with MPTP-induced neuronal loss (Kalaria et al. 1987; Riachi et al. 1988). Since MPP^+

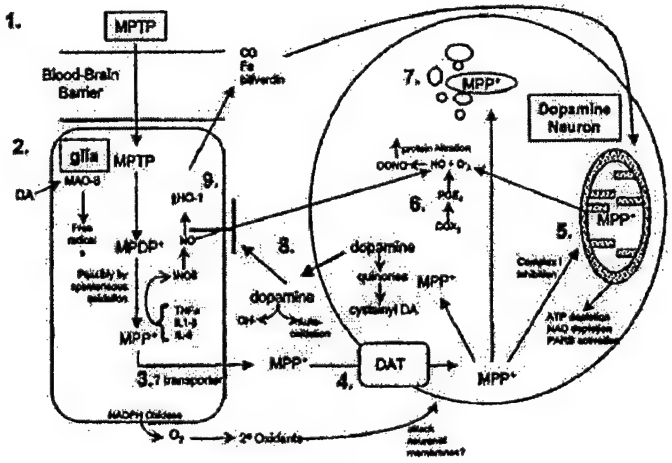

FIGURE 1 Proposed mechanism of MPTP action in the substantia nigra and striatum. The numbers represent each step in the toxification process outlined in this chapter.

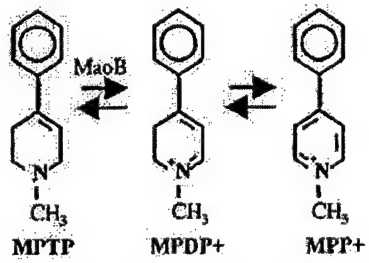

FIGURE 2 The protoxin MPTP is converted by monoamine oxidase B (MaoB) through intermediates to the toxin MPP^+.

cannot be transported through the BBB, (Riachi et al. 1990), this level of toxification/detoxification can provide a first line of defense against exogenous agents.

Step 2. Role of Glia in the Toxification of MPTP

MPTP that is not deprotonated to MPP^+ rapidly enters the brain and is taken up into glial cells by a number of mechanisms including monoamine (Brooks et al. 1989) and glutamate (Hazell et al. 1997) transporters or pH-dependent antiporters (Kopin 1992; Marini et al. 1992). Glia, like endothelial cells, also contain large pools of monoamine oxidases, and also convert MPTP from its protoxin form to MPP^+ (Ransom et al. 1987), in a manner dependent on the presence of MAO-B. A study by Brooks et al. (1989) showed additional support for the role of glial cells in dopaminergic neuronal toxicity, demonstrating that administration of fluoxetine (a serotonergic uptake inhibitor) immediately before systemic injection of MPTP attenuated neurotoxicity. Because fluoxetine did not alter the neurotoxicity of injected MPTP, the site of activation was proven to be extraneuronal, lending credence to the observation that the primary step in MPTP toxicity occurred in the astrocyte.

Once converted to MPP^+ in the astrocyte, MPP^+ stimulates the up-regulation of TNF-alpha, interleukin-1-beta (IL-1β) and interleukin-6 (IL-6) (Youdim et al. 2002; Teismann et al. 2003a) and these, in turn, up-regulate inducible nitric oxide synthese (iNOS) (Hunot et al. 1999). Of the three NOS isoforms present in the brain, endothelial NOS (eNOS), found mainly in the vasculature of the brain, does not contribute to MPTP toxicity (Wu and Przedborski personal communication). In addition, since neuronal NOS (nNOS) knock-out mice show partial protection against the disastrous effects of MPTP administration (Przedborski et al. 1996) in the substantia nigra pars compacta (SNpc), another NOS isoform must also contribute to the neurotoxicity of MPTP. iNOS, a NOS isoform that is minimally expressed in the brain in non-pathological conditions, is highly expressed in the substantia nigra in both Parkinson disease (PD) and in mice (most likely in the microglia) following MPTP treatment (Hunot et al. 1996; Liberatore et al. 1999; Wu et al. 2002; Wu et al. 2003). iNOS produces large amounts of nitric oxide (NO) which is an uncharged, lipophilic molecule (Lancaster 1996), that can freely pass through membranes and travel distances greater than the length of a neuron, up to 300 microns, to do its damage remotely. Thus, under pathological conditions or following MPTP treatment, neurons in the vicinity of the NO molecule are at risk for possible attack by glial-derived reactive nitrogen–related

species. Because minocycline, a second-generation tetracycline antibiotic, can block iNOS induction, (Wu et al. 2002), this step in the toxification process of MPTP presents a point where potential therapeutics may have a significant impact.

Step 3. Release of MPP^+ from Glia

MPP^+ is a polar compound and as such cannot freely exit glial cells. The question of how this compound exits the cell is currently under investigation. Investigators speculate that a specific transporter may actively move this polar molecule out of the glia (Russ et al. 1996; Inazu et al. 2003), however, at present, the specific mechanism remains unknown.

Step 4. Transport of MPP^+ into the Dopaminergic Neuron

Once released into the extracellular space, upon encountering neurons, MPP^+ is taken up into the cell preferentially by the dopamine transporter (DAT). *In situ* analysis has shown that the midbrain contains the highest concentration of dopamine transporters/cell (Cerruti et al. 1993). For this reason, as well as for the selectivity of dopaminergic neurons to many exogenous compounds, the DAT may be a control point in determining differential susceptibility to agents that are known to damage midbrain neurons (Kitayama et al. 1993; Le Couteur et al. 1997), but see also the study by Higuchi et al. (1995). Two groups demonstrated the absolute necessity for the DAT in MPTP toxicity when they examined mice carrying null mutations of the DAT (Gainetdinov et al. 1997; Bezard et al. 1999). In these studies, MPTP-susceptible strains of mice carrying null mutations of the DAT were completely protected from MPTP toxicity.

Step 5. Effects of MPP^+ on Mitochondria within Dopaminergic Neurons

Once in the cell, MPP^+ has several paths: it can enter into mitochondria (step 5) where it interferes with complex I of the electron transport chain (Nicklas et al. 1987; Lander and Schork 1994) or it can be sequestered into cytoplasmic vesicles through the vesicular monoamine transporter (see step 7) (Liu et al. 1992; Del Zompo et al. 1993). Both of these steps have been implicated in processes that either protect or kill the dopaminergic neurons.

MPP^+ enters the mitochondria by the diffusion of this lipophilic cation through the mitochondrial inner membrane. The uptake of MPP^+ into mitochondria is not passive but is actively driven by an electrical gradient within the membrane (a Km of about 5 mM). This active transport was supported by experiments in which valinomycin plus K^+, which collapses the electrochemical mitochondrial gradient, abolished MPP^+ uptake, while agents that specifically collapsed the proton gradient had no effect on MPP^+ uptake (Ramsay et al. 1986; Ramsay and Singer 1986).

Once in the mitochondria, MPP^+ has been implicated in significant alterations of mitochondrial function. MPP^+ inhibits cellular respiration by blocking the mitochondrial electron transport enzyme NADH:ubiquinone oxidoreductase (complex I) (Nicklas et al. 1985; Suzuki et al. 1990) leading to a reduction in cellular ATP. Although this appears to be the major step in blocking mitochondrial function, studies also demonstrate that MPP^+ can directly inhibit complexes III (ubiquinol:ferrocytochrome c oxidoreductase) and IV (ferrocytochrome c:oxygen oxidoreductase or cytochrome c oxidase) of the electron transport chain (Mizuno et al. 1988a,b). The loss of cellular energy has several consequences, including the generation of the oxygen free radicals that rearrange to form hydrogen peroxide. Further catalysis forms hydroxyl radicals.

The energy depletion due to MPP^+'s interference with complex I-III has led to a number of potential therapies. One of the most interesting is the use of Coenzyme Q10 supplementation, as several studies show that orally administering this enzyme can slow the progression of idiopathic PD (Beal 2003; Muller et al. 2003; Shults 2003).

Step 6. Role of Nitration within Dopaminergic Neurons

Although complex I inhibition by MPP^+ is known to reduce the energy production within dopaminergic neurons, it is possible, if not likely, that this is not the direct cause of the observed neuronal death. The damage done within SNpc neurons likely results from compounds generated in the cell, secondary to energy depletion. The formation of the superoxide radical is one example of this process. To establish the role of the superoxide radical in the MPTP neurotoxic cascade of events, a study by Cleeter et al. (1992), showed that MPP^+ inhibits mitochondrial complex 1 activity, which causes an excessive amount of superoxide radicals to form within the neuronal cytosol. Further support came from a study by Przedborski et al. (1992), which demonstrated that over-expression of the copper-zinc form of superoxide dismutase in mice is neuroprotective against the damaging effects of MPTP. Moreover, research by Wu et al. (2003), using the fluorescent tag hydroethidium, provided an *in vivo* demonstration of the presence of the superoxide radical in the MPTP neurotoxic process.

NO, produced in the glial cells, can enter the cytosol of the neuron via simple membrane diffusion. Neither the superoxide radical nor NO are particularly damaging by themselves; however, when the two interact, peroxynitrite ($OONO^-$), one of the most destructive oxidizing molecules, is formed (Ischiropoulos and al-Mehdi 1995; Przedborski

et al. 2000; Przedborski and Vila 2003). This fast-moving molecule as a single entity is hard to detect, however, its handiwork, the nitration of the tyrosine residues of a number of cellular components that include enzymes, transmitters, proteins, fatty acids, and DNA (Radi et al. 2002) can readily be documented.

While many molecules are affected by peroxynitrite, this chapter concentrates on intracellular proteins that are affected both in PD as well as in the MPTP mouse model. One potential target is tyrosine hydroxylase (TH), which is the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects its dense TH-positive fibers to the striatum (Grofova 1979). Because the cell-body rich SNpc contains primarily the soluble form of the TH enzyme, TH is often used as a faithful phenotypic marker for dopaminergic neuron numbers as well as an indicator of dopaminergic neuron loss (Jackson-Lewis et al. 1995).

TH is a tetrameric enzyme composed of four identical subunits. Each subunit carries catalytic activity and catalytic domains have been localized to the carboxy terminals between leucine residue 188 and phenylalanine residue 456. While rodent (rat and mouse) TH contains seventeen tyrosine residues of which fifteen are in the catalytic domain, human TH, although similar, contains only fifteen tyrosine residues of which fourteen are in the catalytic domain (Saadat et al. 1988; Daubner et al. 1993). Tyrosine residues are the keys to the inactivation and nitration of TH, as they are the targets of nitration. At present, researchers speculate that Tyr225 is the most important residue because it lies within the sequence that is targeted for nitration (Przedborski and Jackson-Lewis 1998).

In PD, clinical symptoms appear when about 60–70% of the TH-positive cells in the SNpc have degenerated (Fahn and Przedborski 2000). In addition to the cell loss, brains of Parkinsonian patients show deficits in TH enzyme activity (Ara et al. 1998). Both *in vitro* and *in vivo* studies demonstrate that peroxynitrite impairs TH activity. In mice treated with MPTP, TH nitration seems to occur as early as three hours after MPTP administration. Immunoprecipitation studies using striata from MPTP-treated mice confirm that TH is indeed the nitrated protein. Furthermore, transgenic mice that overexpress human SOD do not show any detectable levels of nitrated striatal TH following MPTP treatment (Ara et al. 1998). Mice deficient in iNOS show less ventral midbrain nitrotyrosine, a fingerprint for tyrosine nitration, after MPTP administration than in their wild-type counterparts (Liberatore et al., 1999). Thus, the inactivation of TH via its nitration following exposure to both peroxynitrite and MPTP is important to the development of PD in humans and to the MPTP neurotoxic process in mice.

Dopamine (DA) is a relatively unstable molecule that is subjected to hydroxyl radical attack (Slivka and Cohen 1985) and autooxidizes in the extracellular space (Hirrlinger et al. 2002). In addition, dopamine can be nitrated within the neuron (LaVoie and Hastings 1999) and therefore may contribute to the degeneration of the cells that contain it as a transmitter. Here, DA is oxidized to DA quinone, which then undergoes a nucleophilic addition via the sulfhydryl group from cysteine, forming 5-cystenyl-DA (Graham 1978). In pathological situations, the up-regulation of the cyclooxygenase-2 (COX-2) enzyme facilitates the oxidation of DA to 5-cystenyl-DA(Hastings 1995; O'Banion 1999). The relationship of 5-cystenyl-DA to neurodegeneration in PD as well as to the degeneration of DA neurons seen in the MPTP mouse model was recently investigated using a combination of immunocytochemistry (PD brains) or a combination of immunocytochemistry and HPLC (MPTP studies). In both PD brains and ventral midbrain from MPTP-treated mice, COX-2 enzyme activity and protein levels were significantly higher than in controls. Robust COX-2 immunostaining was also noted in both the human and mouse brains where the enzyme appeared to be confined to the cytosol of dopaminergic neurons (Teismann et al. 2003b). Furthermore, inhibition of the COX-2 response to MPTP prevented the rise in protein cysteinyl dopamine that occurred in mice following the administration of MPTP (Teismann et al. 2003b).

Peroxynitrite is formed from NO and the superoxide radical inside the neuron, and as such poses a serious threat to intracellular components such as mitochondria. For example, the nitration of manganese SOD (MnSOD), the primary mitochondrial antioxidant, was detected both *in vitro* (Quijano et al. 2001) and during inflammatory responses *in vivo* (MacMillan-Crow et al. 1996; Aulak et al. 2001). Here, nitration was proven to be site-specific in that it is tyrosine 34 (Tyr^{34}) among the tyrosine residues that nitrated. In the MPTP mouse model, overexpression of human MnSOD localized to mitochondria prevented the accumulation of 3-nitrotyrosine, the faithful fingerprint of peroxynitrite-mediated nitration (Klivenyi et al. 1998). Several other mitochondrial components such as NADH: ubiquinone reductase (Complex I) (Riobo et al. 2001), cytochrome c (Cassina et al. 2000), aconitase, ATPase and VDAC (voltage dependent anion channel) (Radi et al. 2002) are nitrated following exposure to peroxynitrite. Whether these are nitrated in PD and in the MPTP mouse model has yet to be determined.

Step 7. Sequestration of MPP+ within the Dopaminergic Neuron

The vesicular monoamine transporter VMAT2, is a proton-dependent transporter that sequesters monoamine neurotransmitters from free cytoplasmic space into synaptic vesicles (Miller et al. 1999a). Like the monoamines, MPP^+ can be transported by the VMAT into these vesicles, and as such, can be prevented from entering the mitochondria

where it can inhibit complex I. Investigators postulate that this sequestration may be a mechanism for attenuating the effects of any number of monoaminergic toxins. Support for this hypothesis comes from analyses in mice containing partial or complete deletions of VMAT2 and from human studies of VMAT expression. In parkinsonian humans, immunocytochemical localization of VMAT demonstrates reduced expression in striatum, paralleling the reductions seen in DAT. In fact, the relative expression of VMAT2, compared to that of DAT, may allow one to predict if and which dopamine neurons may be lost in PD (Miller et al. 1999b). In animal studies, mice heterozygous for VMAT2 and exposed to MPTP were examined for markers of dopaminergic neuron toxicity, including striatal dopamine content, the levels of DAT protein, as well as for a secondary marker of neurotoxicity, the expression of glial fibrillary acidic protein (GFAP) mRNA. In all parameters measured, VMAT2 +/- mice were more sensitive to MPTP-induced toxicity than their wild-type littermates (Gainetdinov et al. 1998). Further examination of these mice revealed that heterozygous VMAT2 mice, in addition to the loss of striatal markers, also had increased SNpc cell loss following administration of MPTP (Takahashi et al. 1997). These studies suggested an important role for VMAT2 in potentiating the effects of MPTP. Conversely, cells transfected to overexpress a greater density of VMAT2 were converted from MPP^+ sensitive to MPP^+ resistant cells (Liu et al. 1992). These studies suggested an important role for VMAT2 in potentiating or allaying the effects of MPTP.

Alpha-synuclein is another molecule relevant to the development of PD in humans and to the neurotoxic process in the MPTP mouse model of PD that is susceptible to nitration because of the presence of tyrosine residues. Historically, synucleins are vertebrate-specific cytosolic proteins that contain about 127–140 residues that have a unique 11-residue repeat that occurs in five to seven copies, accounting for roughly half of their structure and no structural domains. Four proteins, alpha, beta, and gamma synuclein and synoretin make up this family of proteins. Only two proteins in this family, alpha and beta, are synthesized in relatively large amounts in the brain and are highly expressed in presynaptic nerve terminals (Schluter et al. 2003). Synucleins account for about 1% of brain proteins and to date their functions are still unknown. Mutations in alpha-synuclein are associated with a familial form of PD (Polymeropoulos et al. 1997) that is readily indistinguishable from the more common sporadic form of the disease. The interaction between WT alpha-synuclein and mutant alpha-synuclein may enhance the ability of the different alpha-synucleins to interact with other cellular proteins to form aggregates (Conway et al. 1998).

One of the hallmarks of PD is the presence of Lewy bodies within neurons in the SNpc. Lewy bodies are both ubiquitin and alpha-synuclein positive. Since alpha-synuclein is the only synuclein present in Lewy bodies, it has to be determined whether this molecule is toxic or whether it is just a by-product of cellular metabolism in a pathological situation. A number of cellular proteins have been found to be nitrated in PD tissues (Ischiropoulos and al-Mehdi 1995), which was taken as evidence that nitrating agents such as peroxynitrite engaged in nitration reactions here. Specific antibodies that recognize nitrated alpha-synuclein have demonstrated that alpha-synuclein is the protein that is nitrated in Lewy bodies in a number of disease states including PD (Giasson et al. 2000b; Giasson et al. 2000a). Furthermore, alpha-synuclein inclusions in tissues from PD patients were strongly labeled with antibodies that recognize the faithful fingerprint of peroxynitrite-induced nitration, 3-nitrotyrosine (Souza et al. 2000). Both *in vitro* studies and the MPTP mouse model were used to prove that tyrosine residues in the alpha-synuclein molecule are indeed the targets of nitration and that peroxynitrite is indeed the culprit. In HEK 293 cells transfected to overexpress human alpha-synuclein and that were exposed to peroxynitrite, a nitrated band that corresponded to the molecular mass of alpha-synuclein was noted (Przedborski et al. 2001). In the MPTP mouse model, immunoprecipitation studies using striatum and ventral midbrain from treated mice showed that alpha-synuclein was nitrated as early as four hours after MPTP administration. In contrast, beta-synuclein was not nitrated in either situation (Przedborski et al. 2001).

Step 8. Release of Dopamine from Intracellular Stores

A second consequence of the depletion of cellular ATP is the release of dopamine from intracellular stores (Schmidt et al. 1984; Ofori and Schorderet 1987; Rollema et al. 1988; Lau et al. 1991; Schmidt et al. 1999). Once released into the extracellular space, the enzymatic oxidation of dopamine results in the rapid formation of hydroxyl radicals. It is clear that the presence of free radicals can lead to membrane damage and subsequent cell death. That dopamine rapidly auto-oxidizes and contributes to neurotoxicity always leads to the controversial topic of L-dopa therapy in PD. Simply stated, one can question whether the therapy that best treats the symptoms of PD may also exacerbate the disease. In support of this hypothesis, Whone and colleagues showed that the progression of PD using PET scanning was greater in patients treated with L-dopa than those treated with the dopamine agonist ropinirole (Whone et al. 2003). However, other studies do not support this hypothesis (Fornai et al. 2000; Melamed et al. 2000), and for this reason, the question of L-dopa toxicity has yet to be resolved.

While the above question is still not settled, the formation of hydroxyl radicals apart from direct dopamine oxida-

tion can also modulate several other processes that can lead to cell death, including the fragmentation of DNA (Walkinshaw and Waters 1995) and inhibition of Na+, K+-ATPase activity (Khan et al. 2003). Additional sites of hydroxyl radical formation may occur as a result of interactions with neuromelanin (D'Amato et al. 1986) as well as with cellular iron (Jellinger 1999), each of which could contribute to its neurotoxicity.

Step 9. A Second Role for Glial Cells?

Based upon our hypothesis of the mechanism(s) of MPTP-induced cell death (figure 1), a dramatic interplay occurs between neurons and the non-neuronal milieu. As discussed earlier in this chapter (Step 2), the astrocytes are necessary for the bioactivation of MPTP into its toxic metabolite, MPP^+. The glial cells, in addition to their toxifying function, also are believed to play a significant role in neuronal protection. A recent report, using *in vitro* chimeric cell cultures, has demonstrated that the toxicity of MPTP is determined by the response of the glial cells following MPP^+ intoxication (Smeyne et al. 2001) and numerous *in vitro* studies support this data (Di Monte et al. 1992; Forno et al. 1992; Di Monte et al. 1996).

Glial cells contribute directly to the toxic effects of MPTP through several mechanisms, including the mediation of free radical formation and damage by induction of nitric oxide synthase (iNOS) (Hirsch et al. 1998; McGeer and McGeer 1998; McNaught and Jenner 1999). Administration of MPTP leads to a rapid gliosis (Schneider and Denaro 1988), which subsequently increases production and releases iNOS (Zietlow et al. 1999). In a model of iNOS action that extends the role of glia, Hirsch and Hunot (2000) suggest that MPTP acts directly on the induction of cytokines that activates iNOS. iNOS is then released from the glial cells to directly damage the dopaminergic neurons. Thus, differential expression of iNOS may underlie some of the strain specific responses to MPTP seen in mice, and perhaps, the differential sensitivity to different environmental toxins in humans.

In addition to inducing and modulating cytokines, dopamine in the extracellular space can induce a number of different molecules that are involved in oxidative stress. One of these molecules, heme oxygenase-1 (Fernandez-Gonzales et al. 2000), the rate limiting enzyme in heme degradation, plays a critical role in heme and iron homeostasis (Schipper et al. 1998b; Maines 2000). Several isoforms of heme oxygenase have been identified (reviewed in Elbirt and Bonkovsky 1999), each of which converts heme to bilirubin and carbon monoxide, while at the same time releasing iron into the cellular milieu (Maines 1997). Further support for the importance of this molecule is that heme oxygensase-1 is elevated in astrocytes of Parkinsonian patients (Schipper et al. 1998a). In addition, the brains of heme oxygenase-1 null mice show excessive iron deposits, increased sensitivity to oxidative stress, and chronic inflammation (Poss and Tonegawa 1997). Moreover, astrocytes in the striatum of MPTP-treated mice show increases in heme oxygensase-1 as early as six hours after the administration of MPTP (Fernandez-Gonzales et al. 2000). On the flip-side, overexpression of heme oxygensase-1 leads to a reduced damage in the presence of free radicals (Maines 1997) which is why investigators have postulated the induction of heme oxygensase-1 as a potential therapy for PD. However, based on the breakdown of heme, which leads to the formation of biliverdin and carbon monoxide as well as free iron, it is possible that in the specific environment of the SNpc, heme oxygensase-1 can act counterintuitively and lead to further neurotoxicity (Hansen 1994; Schipper 1999). The breakdown products of heme induced by heme oxygensase-1 also may act as mitochondrial toxins, leading to a feed-forward loop that eventually leads to cell death.

In addition to participating in cellular toxicity, astrocytes, either in the substantia nigra or striatum, may also act as a protective agent through several mechanisms, including their ability to act as "cellular buffers" and by producing neurotrophic factors. Several studies show that astrocytes can aid in neuronal protection thorough the synthesis and release of the free-radical scavenger glutathione and/or its precursors glutamate, cysteine, and glycine (Drukarch et al. 1998; Dringen et al. 1999). Unlike neurons, glia can generate this neuroprotectant through the biochemical pathways that use cysteine to produce GSH (Sagara 1993 #1117; Wang and Cynader, 2000). Since GSH levels are lower in the SNpc of PD patients, the local astrocytes in the substantia nigra may serve this important function. The efficiency of glial cells in producing or in maintaining levels of glutathione in different strains of mice (Hatakeyama et al. 1996) may be an important factor in the pathogenesis of dopaminergic neuron loss in experimental models of PD and may provide a therapeutic target for neuroprotection.

In addition to providing the precursors for redox modulating compounds such as glutathione, astrocytes also produce a number of neurotrophic factors (Schaar et al. 1993; Schaar et al. 1994; Nakajima et al. 2001). Several neurotrophins support dopaminergic neurons following MPTP or MPP^+ intoxication (Nagatsu et al. 2000). These factors include BDNF (Spina et al. 1992; Frim et al. 1994; Tsukahara et al. 1995), GDNF (Cheng et al. 1998; Date et al. 1998), FGF (Otto and Unsicker 1994) and EGF (Hadjiconstantinou et al. 1991). Neurotrophins act to prevent cell death through a number of mechanisms including interference with the intrinsic cell death programs (Schabitz et al. 2000; Heaton et al. 2003) and modulating oxidative stress (Spina et al. 1992; Kirschner et al. 1996; Skaper et al. 1998; Gong et al. 1999; Petersen et al. 2001).

III. CONCLUSIONS

The discovery that MPTP, which is structurally similar to a number of commonly used herbicides and pesticides, can induce specific loss of substantia nigra neurons in many vertebrate species, from humans to mice, has lead to the development of a useful model of Parkinson disease. In mice, MPTP demonstrates differential toxicity that is dependent on the strain of animal examined (Sonsalla and Heikkila 1988; Muthane et al. 1994; Hamre et al. 1999). This finding supports the hypothesis that the loss of substantia nigra neurons in Parkinson disease may result from a genetic sensitivity to a number of environmental agents (Veldman et al. 1998; Stoessl 1999). In a recent study, the chromosomal loci containing the genetic sequences responsible for this sensitivity was identified on the telomeric end of mChr.1 (Cook et al. 2003). Further studies into the genetic and biochemical pathways involved in MPTP toxicity will lead to a better understanding of idiopathic Parkinson disease and provide clues to novel targets for therapeutic interventions.

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THE MPTP MODEL OF PARKINSON'S DISEASE

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Abstract

The biochemical and cellular changes that occur following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are remarkably similar to that seen in idiopathic Parkinson's disease. In this review, we detail the molecular activities of this compound from peripheral intoxication through its various biotransformations. In addition, we detail the interplay that occurs between the different cellular compartments (neurons and glia) that eventually consort to kill substantia nigra pars compacta neurons.

Parkinson's disease (PD), was first described in a paper entitled "An Essay on the Shaking Palsy", in 1817 by Dr. James Parkinson in [109]. PD is a progressive neurological disorder that strikes approximately 2% of the "over 50" population [20]. Current estimates from the American Parkinson's Disease Foundation put the number of American citizens suffering from this disease at greater than 1.5 million persons. At this time, PD is the 3rd most prevalent neurodegenerative disorder, following Alzheimer's disease and dementia without Lewy body disease. Since the disease incidence increases with age, it is likely that the number of people suffering from PD will rise as improved healthcare lengthens the average lifespan.

The main anatomical feature of PD is the decrease in number of neuromelanin-containing neurons located in the midbrain substantia nigra pars compacta (SNpc). These dopaminergic neurons project to the striatum as well as a number of other subcortical regions [161]. PD symptoms first manifest when approximately 60% of the SNpc neurons have already died [39] and 70% of dopamine responsiveness disappears [83]. Because the progression of cell loss is thought to occur over a somewhat protracted period of time in a defined spatiotemporal manner [18,104], the onset of Parkinson's disease symptoms is often insidious.

At this time, it is unclear as to how much of the disease results from a purely environmental factor, a strict genetic causation, or a combination of the two [24,146]. Most epidemiological studies conclude that less than 10% PD has a strict familial etiology [110]. This includes a small number of familial parkinsonian patients with polymorphisms in the α-synuclein gene [112] (reviewed by Lundvig et al (this issue)) as well as patients with early onset PD that have recessive mutations in the Parkin gene, mapping to the long arm of chromosome 6 (6q25.2-q27). These the pathogenicity of these proteins is discussed in a review by Burke [9].

Familial PD has also been associated with human chromosome 2p13 and 4p polymorphisms [38]. The PD linked to this locus more closely resembles that of idiopathic PD, although like the □-synuclein protein, this unknown protein has very low penetrance. Although alterations in the proteins coded for by these loci may lead to an understanding of the molecular processes that occur in idiopathic PD, no mutations as of yet have been reported in aged-onset idiopathic PD [56,138].

Since the majority of PD patients have no identifiable genetic mutation, important information regarding the pathophysiology of PD may be learned through the study of animal models. At this time, several animal models have been developed to study the underlying the mechanisms that lead to the development of experimental PD. One of the earliest models made use of a lesion of nigrostriatal pathway in which fibers emanating from the substantia nigra proceeded to the striatum rostrally through the medial forebrain bundle, [6,31,80]. Other models have used chemical lesions. One example is the use of 6-OHDA, a neurotoxin that when injected into the striatum causes a retrograde degeneration of dopaminergic neurons in the SNpc (reviewed in [22,54,107,137]). Another model of experimental PD utilizes the properties of selective neurotoxins, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

The sequalae of degeneration that occurs following administration of MPTP in animals has provided a useful model of Parkinsonism because it induces pathologies similar to that seen in man. Interestingly, the use of MPTP may be one of the few cases in which the effects of a neurotoxin were discovered in humans first, followed by development of an animal model. Although there were early reports of neurotoxicity to this compound, its use as a research tool became prevalent in the early 1980's following the identification of a number of Northern California heroin users who presented at various emergency rooms with symptoms

indistinguishable from those of Parkinson's disease [10,75]. A complete history of these cases is presented in "The Case of the Frozen Addicts" [76].

Although MPTP was first identified as a parkinsonian agent in humans, it has been demonstrated to exert similar effects in a number of other primates [63,70,157], as well as in cats, and in several rodents. In rodents, it has been shown that only specific strains of mice are sensitive to the administration of MPTP [46,94,124,148]. MPTP structurally resembles a number of known environmental agents, including well-known herbicides such as paraquat [23] and garden insecticides/fish toxins such as rotenone [89] that have been shown to induce dopamine cell degeneration [4,7,13,153], although mechanistically, the actions of each are likely different [23]. Further studies of each of mechanism of each of these toxins may lead to a unified pathway that underlies these toxins.

Mechanism of MPTP action

There are many points systemically where MPTP can affect the dopaminergic system (Figure 1). The first point of potential modulation of any exogenous compounds neurotoxicity is the blood-brain-barrier (BBB). The BBB is composed on tight-junctioned endothial cells that make up the microvasculature of the brain in tight opposition with the end feet of glial processes. MPTP first is metabolized by the enzyme MAO-B to 1-methyl-4-phenyl-2, 3-dihydropyridium ($MPDP^+$) that then deprotonates to generate MPTP the corresponding pyridium species, MPP^+ (Figure 2). Endothelial cells in the microvasculature that make up the BBB contain monoamine oxidases; and several studies have correlated levels of monoamine oxidases with MPTP-induced neuronal loss [64,125]. Since MPP^+ cannot be transported through the BBB, [123], this level of toxification/detoxification can provide a first line of defense against exogenous agents.

Role of Glial cells (part 1)

MPTP that is not converted to MPP^+ in the periphery rapidly enters the brain where it is processed into glial cells by a number of mechanisms including monoamine [8] and glutamate [50] transporters or pH-dependent antiporters [69,87]. Glia, like the previously mentioned endothelial cells, also contain large pools of monoamine oxidases, and also convert MPTP from its protoxin form to MPP^+ [122]. Additional support for the role of glial cells in dopaminergic neuronal toxicity was shown by Brooks et al [8] who demonstrated that administration of a serotonergic uptake inhibitor, fluoxetine, immediately before systemic injection of MPTP altered the observed neurotoxicity. Since fluoxetine did not alter the neurotoxicity of injected MPTP, it was proven that the site of activation was extraneuronal, lending credence to the observation that the primary step in MPTP toxicity occurred in the astrocyte.

Once converted to MPP^+ in the astrocyte, MPP^+ stimulates the up-regulation of TNF-α, interleukin-1β (IL-1β) and IL-6 [152,160] and these, in turn, up-regulate inducible nitric oxide synthese (iNOS) [57]. Of the three NOS isoforms present in the brain, endothelial NOS (eNOS), found mainly in the vasculature of the brain, is not altered following to MPTP toxicity [98]. In addition, since nNOS (neuronal NOS) knockout mice show partial protection against the MPTP toxicity in the substantia nigra pars compacta (SNpc) [116], another NOS isoform must also contribute to the neurotoxicity of MPTP. iNOS, a NOS isoform that is minimally expressed in the normal brain, has been shown to be upregulated in the substantia nigra's microglia in both Parkinson's disease (PD) and in mice following MPTP treatment [68,81,158,159]. iNOS produces large amounts of the uncharged and lipophillic molecule nitric oxide (NO) and as such can freely pass through membranes and travel distances greater than the length of a neuron [73]. Thus, following MPTP treatment, neurons in the vicinity of the NO molecule are put at risk for possible attack by glial-derived reactive nitrogen–related species. Since iNOS induction can be

blocked by the antibiotic minocycline [158], this step in the toxification process of MPTP presents a point where potential therapeutics may have a significant impact.

Since MPP^+ is a polar compound, it cannot freely exit from its glial environs. It has been suggested that there may be a specific transporter that actively moves this polar molecule out of the glia [58,127], however, at present, this specific mechanism remains unknown.

Once MPP+ is released into the extracellular space, MPP^+ is taken up into dopaminergic cells by the dopamine transporter (DAT). Since midbrain neurons contains the highest concentration of dopamine transporters/cell [11], the DAT may be a control point in determining how susceptible midbrain neurons are to exogenous agents [67,79] (but see also [52]. The requirement for the DAT in relation to MPTP toxicity was demonstrated by two groups examining mice carrying null mutations of the DAT [5,36]. In these studies, MPTP-susceptible strains of mice carrying null mutations of the DAT were completely protected from MPTP toxicity.

Role of Dopaminergic neurons

Once in the cell, MPP^+ can move through several cellular compartments: it can enter into mitochondria where it interferes with complex I of the electron transport chain [74,103] or it can be sequestered into cytoplasmic vesicles by actions of the vesicular monoamine transporter [21,82].

MPP^+ enters the mitochondria by the diffusion through the mitochondrial inner membrane. The uptake of MPP^+ into mitochondria is actively driven by a membrane electrical gradient ($Km \approx 5$ mM). This active transport was supported by experiments in which valinomycin plus potassium, which collapses the mitochondrial electrochemical gradient, eliminated MPP^+

uptake, while agents which collapsed this proton gradient had no effect on MPP^+ uptake [120,121].

Once in the mitochondria, MPP^+ inhibits cellular respiration through the blockade of the electron transport enzyme NADH:ubiquinone oxidoreductase (complex I) [102,149]. Blockade of this complex leads to a reduction in cellular ATP. Although this appears to be the major step in blockade of mitochondrial function, studies have shown that MPP^+ can also directly inhibit complexes III (ubiquinol:ferrocytochrome c oxidoreductase) and IV (ferrocytochrome c:oxygen oxidoreductase or cytochrome c oxidase) of the electron transport chain [95,96]. The loss of cellular energy through each of these pathways has several consequences, including the generation of the oxygen free radicals that rearranges to form hydrogen peroxide. Further catalysis leads to the formation of hydroxyl radicals.

Based upon the finding that MPP^+ depletes cellular energy due to interference with complex I-III, and as such may be related to the etiology of human PD, a number of potential therapies have been examined. One promising study has used Coenzyme Q10 supplementation, where oral administration of this compound in fairly high doses has been observed to slow the progression of the disease [2,97,139].

Although complex I inhibition by MPP^+ reduces energy production within dopaminergic neurons, if is likely that this is not the immediate cause of the SNpc neuronal death. The damage done within these dopaminergic neurons is likely to result from compounds generated in the cell, secondary to energy depletion. The formation of the superoxide radical is one example of this process. Cleeter et al [14], showed that MPP^+, following inhibition of mitochondrial complex 1 activity, formed an excessive amount of superoxide radicals within the neuronal cytosol. Further support for the role of superoxide radicals came from Przedborski et al. [117], who demonstrated

that over-expression of the copper-zinc form of superoxide dismutase in mice is neuroprotective against the damaging effects of MPTP. Moreover, Wu et al [159], using the fluorescent tag hydroethidium, provided an in vivo demonstration of the presence of the superoxide radicals following MPTP intoxication.

NO, produced and released by glial cells, can enter the cytosol of the neuron via simple membrane diffusion. At this point, the superoxide radical and NO, which are not particularly damaging by themselves; can interact to form peroxynitrite ($OONO^-$), one of the most destructive oxidizing molecules [59,115,118]. Although difficult to detect due to its rapid processing, the nitration of the tyrosine residues of a number of cellular components include enzymes, transmitters, proteins, fatty acids and DNA can easily be identified [119].

One potential target of $OONO^-$ is tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects fibers to the striatum [44]. Since it has been shown that the cell-body rich SNpc primarily contains the soluble form of the TH enzyme, it is often used as the phenotypic marker for dopaminergic neuron numbers and can be measured both by biochemical and immunohistochemical methods to determine neuron loss [46,60].

Clinical symptoms are first thought to appear when about 60-70% of the TH-positive cells in the SNpc have degenerated [30]. Brains of parkinsonian patients also show deficits in TH enzyme and TH enzyme activity [61]. Both *in vitro* and *in vivo* studies demonstrate that peroxynitrite impairs TH activity [71]. In MPTP-treated mice, TH nitration occurs soon after MPTP administration. Furthermore, transgenic mice that overexpress human SOD do not show any detectable levels of nitrated striatal TH following MPTP treatment [1]. Mice deficient in iNOS show less ventral midbrain nitrotyrosine, a fingerprint for tyrosine nitration, after MPTP

administration than in their wild-type counterparts [81]. Thus, the inactivation of TH via its nitration following exposure to both peroxynitrite and MPTP appears to be an important process in the development of PD in humans and to the MPTP neurotoxic process in mice.

Dopamine (DA) is a relatively unstable molecule that is subject to both hydroxyl radical attack [141] and autooxidation in the extracellular space [53]. In addition to extraneuronal effects, dopamine can also be nitrated intracellularly [78] and therefore may contribute to the degeneration of the cells that contain this neurotransmitter. In this process, DA is oxidized to DA quinone, which then undergoes a nucleophilic addition via the transfer of a sulfhydryl group from cysteine, to form 5-cystenyl-DA [43]. In pathological situations, the oxidation of DA to 5-cystenyl-DA is facilitated by the up-regulation of cyclooxygenase-2 (COX-2) [48,105]. The role of 5-cystenyl-DA in the development of PD as well as to the degeneration of DA neurons seen in the MPTP mouse model was examined in a variety of studies. For example, it was observed that COX-2 immunostaining was robust in the human and mouse dopaminergic neurons [151]. Additionally, COX-2 enzyme activity and protein levels in both PD brains and ventral midbrain from MPTP-treated mice were found to be significantly higher than in controls. Inhibition of the COX-2 response to MPTP, however, prevented the rise in protein cysteinyl dopamine that was seen to occur in mice following the administration of MPTP [151].

The vesicular monoamine transporter VMAT2, is a proton-dependent transporter that sequesters monoamine neurotransmitters from free cytoplasmic space into synaptic vesicles [93]. Since it structurally resembles monoamines, MPP^+ can be transported by the VMAT into these vesicles, thus being prevented from entering the mitochondria where it can inhibit complex I. This sequestration has been hypothesized to be as a potential mechanism for reducing the deleterious effects of any number of monoaminergic toxins. Support for this hypothesis comes

from analyses in mice containing partial or complete deletions of VMAT2 as well as from human studies of VMAT expression. In parkinsonian humans, immunocytochemical localization of VMAT showed reduced expression in striatum, similar to that seen in the DAT. In fact, Miller et al suggested that the relative expression of VMAT2, compared to that of DAT, may allow one to predict if and which dopamine neurons may be lost in PD [92] (see the possible application of this hypothesis in Faherty et al, this issue). In animal studies, VMAT2(+/-) mice exposed to MPTP were examined for markers of dopaminergic neuron toxicity, including dopamine content and DAT protein in the stiatum, as well as expression of glial fibrillary acidic protein (GFAP) mRNA. In all parameters measured, VMAT2(+/-) mice were more sensitive than their wild-type littermates to MPTP-induced toxicity [37]. Further examination of these mice revealed that VMAT2(+/-) mice, following administration of MPTP, also had increased SNpc cell loss [150]. These studies suggested an important role for VMAT2 in potentiating the effects of MPTP. Using an in vitro system, cells transfected to overexpress a greater density of VMAT2 were converted from MPP^+ sensitive to MPP^+ resistant cells [82]. These studies suggested an important role for VMAT2 in modulating the effects of MPTP.

Another molecule of interest relevant to the development of PD in humans and to the neurotoxic process in the MPTP mouse model of PD is α-synuclein. Synucleins are cytosolic proteins that contain 127-140 residues that have a unique 11-residue repeat that occurs in 5-7 copies which accounts for roughly one-half of their structure and no structural domains (see review by Lundvig et al, this issue). Four proteins, α, β and γ synuclein as well as synoretin make up this family of proteins of which only two, α and β, are synthesized in relatively large amounts in the brain (making up approximately 1% of total brain protein). These protein are generally found in abundance in presynaptic nerve terminals [134]. Mutations in α-synuclein

have been associated with a familial form of PD [112] that is readily indistinguishable from the more common sporadic form of the disease. It is thought that the interaction between WT and mutant α-synucleins may enhance the ability of these proteins to interact with other non-synuclein cellular proteins to form aggregates [15].

The presence of Lewy bodies within neurons in the SNpc is one of the characteristic pathologies seen in PD. Lewy bodies are both ubiquitin and α-synuclein immunopositive. Since α-synuclein is the only synuclein present in Lewy bodies, it has to be determined whether this molecule is toxic or whether it is just a by-product (tombstone) of cellular metabolism in a pathological situation. A number of cellular proteins have been found to be nitrated in PD brains [59] and specific antibodies that recognize nitrated α-synuclein have been used to demonstrate that alpha-synuclein is the protein that is nitrated in Lewy bodies [40,41]. Furthermore, α-synuclein inclusions in tissues from PD patients have been shown to be strongly labeled with antibodies that recognize the hallmark of peroxynitrite-induced nitration, 3-nitrotyrosine [144]. Two lines of evidence support these conclusions. In vitro studies using HEK 293 cells transfected to overexpress human α-synuclein that were exposed to peroxynitrite, showed a nitrated band that corresponded to the molecular mass of α-synuclein was noted [114]. In vivo, using the MPTP mouse model, immunoprecipitation studies of midbrain and striatum showed that α-synuclein was nitrated as early as 4 hours after MPTP administration. Sepcificity for this form of synuclein was demonstrated by the observation that β- synuclein was not nitrated in either situation [114].

Another consequence of the cellular ATP depletion is the abnormal release of DA from intracellular stores [77,106,126,135,136]. Once DA is released into the extracellular space, the enzymatic oxidation of DA results in the formation of hydroxyl radicals. That dopamine rapidly

auto-oxidizes and contributes to neurotoxicity always leads to the controversial topic of L-dopa therapy in PD. Simply stated, one can question whether the therapy that best treats the symptoms of PD may also exacerbate the disease. In support of this hypothesis, Whone et al showed that the progression of PD using PET scanning was greater in patients treated with L-dopa than those treated with the dopamine agonist ropinerole [86,156]. However, other studies do not support this hypothesis [33,91], and for this reason, this question has yet to be sorted out.

Additional sites of hydroxyl radical formation may occur as a result of interactions with neuromelanin [17] as well as with cellular iron [62], each of which could contribute to its neurotoxicity.

Glial Cells (Part 2)

The mechanism(s) of MPTP-induced cell death (Figure 1), show a great amount of crosstalk between the neurons and the non-neuronal milieu. Previously, we discussed how the astrocytes are necessary for the conversion of MPTP to MPP^+. In addition to this function, astrocytes are also believed to play a significant role in neuroprotection. A study using chimeric SN cell cultures, has demonstrated that the differential toxicity of MPTP in mouse strains is determined by the response of the glial cells [142]. This work is supported by other in vitro studies [25,26,34].

As discussed earlier in this review, glial cells directly contribute to the toxicity seen following administration of MPTP through several mechanisms, including the mediation of free radical formation and damage by induction of nitric oxide synthase (iNOS) [55,88,90]. In addition to the induction and modulation of cytokines, the presence of dopamine in the extracellular space can induce a number of different molecules that are involved in oxidative stress. One of these molecules, hemeoxygenase-1 (HO-1), the rate limiting enzymes in heme

degradation, has been shown to play a critical role in iron and heme homeostasis [85,133]. It is well known that alterations in brain iron are seen in PD brains [3,65,72]. Several isoforms of hemeoxygensase have been identified (reviewed in [29], each of which converts heme to bilirubin and carbon monoxide, while at the same time releasing iron into the cellular milieu [84]. Further support for the importance HO-1 is that it is elevated in astrocytes of parkinsonian patients [132]. In striatal astrocytes, HO-1 elevation occurs as early as 6 hours following administration of MPTP [32]. In addition, brains of HO-1 null mice show excessive deposition of iron, increased sensitivity to oxidative stress and chronic inflammation [113]. On the flip-side, overexpression of HO-1 leads to a lessening of damage that has been observed in the presence of free radicals [84]. For this reason, modulation of HO-1 has been postulated as a potential therapy for PD. However, based on the breakdown of heme, which leads to the formation of bilivirdin, carbon monoxide and free iron, it is possible that in the specific environment of the SN, HO-1 can act counterintuitively and lead to a furthering of neurotoxicity [47,131]. It is also possible that the breakdown products of heme induced by HO-1 act as mitochondrial toxins leading to a feed-forward loop that eventually leads to cell death.

In addition to acting as participants in cellular toxicity, astrocytes, either in the substantia nigra or striatum, may also act as a protective agent through several mechanisms including their ability to act as a "cellular buffers" and production of neurotrophic factors. Several studies have shown that astrocytes synthesis and release the free-radical scavenger glutathione and/or its precursors glutamate, cysteine and glycine [27. This function is specific to astrocytes and not neurons- as they are able to generate this neuroprotectant thorough the biochemical pathways that use cystine as well as cysteine for the production of GSH [Sagara, 1993 #1117,28,155]. Since GSH levels are lower in the SNpc of PD patients, the local SNpc astrocytes may serve this

critical function. The efficiency of glial cells in producing or in maintaining levels of glutathione in different strains of mice [49] may be an important factor in the pathogenesis of dopaminergic neuron loss in experimental models of PD and may point to this pathway as a therapeutic target for neuroprotection.

In addition to providing the precursors for redox modulating compounds such as glutathione, astrocytes have also been shown to produce a number of neurotrophic factors [101,128,129]. Several neurotrophins have been shown to protect dopaminergic neurons from cell death following MPTP or MPP^+ intoxication [100]. These factors include BDNF [35,145,154], GDNF [12,19], FGF [108] and EGF [45]. Neurotrophins act to prevent cell death through a number of mechanisms including modulation of oxidative stress [42,66,111,140,145] as well as interference with the intrinsic cell death programs [51,130].

Conclusions.

MPTP, which is structurally similar to a number of commonly used herbicides and pesticides, can induce specific loss of substantia nigra neurons in many vertebrate species, from man to mouse. Studies using this toxin have lead to the development of useful animal models of Parkinson's disease. In mice, MPTP demonstrates differential toxicity that is dependent on the strain of animal examined [46,99,143](see also XXXXX in this issue). This finding supports the hypothesis that the loss of substantia nigra neurons in Parkinson's disease may result from a genetic sensitivity to a number of environmental agents [24,147]. Recently, the chromosomal loci containing the genetic sequences contributing to this sensitivity to neuronal loss [16] in mice has been localized on the telomeric end of mChr.1 [16]. Further studies into the genetic as well as into the biochemical pathways involved in MPTP toxicity will lead to a better understanding

of idiopathic Parkinson's disease as well as provide clues to novel targets for therapeutic interventions.

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Minireview

MPTP and SNpc DA Neuronal Vulnerability : Role of Dopamine, Superoxide and Nitric Oxide in Neurotoxicity

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Abstract.

Parkinson disease (PD) is a common neurodegenerative disease of unknown origin that is characterized, mainly, by a significant reduction in the number of dopamine neurons in the substantia nigra pars compacta of the brain and a dramatic reduction in dopamine levels in the corpus striatum. For reasons that we do not know, the dopamine neuron seems to be more vulnerable to damage than any other neuron in the brain. Although hypotheses of damage to the dopamine neuron include oxidative stress, growth factor decline, excitotoxicity, inflammation in the substantia nigra pars compacta (SNpc) and protein aggregation, oxidative stress in the nigrostriatal dopaminergic system garners a significant amount of attention. In the oxidative stress hypothesis of PD, superoxide, nitric oxide and dopamine all conspire to create an environment that can be detrimental to the dopamine neuron. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the tool of choice for investigations into the mechanisms involved in the death of dopamine neurons in PD, has been used extensively in attempts to sort out what happens in and around the dopamine neuron. Herein, we review the roles of dopamine, superoxide and nitric oxide in the demise of the dopamine neuron in the MPTP model of PD as it relates to the death of the dopamine neuron noted in PD.

Introduction

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability, these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) whose fibres project to the corpus striatum. There are, at present, 1 million PD patients in the United States alone, with 50,000 newly diagnosed cases each year (Fahn and Przedborski, 2000). These cases include both familial and sporadic PD, of which sporadic PD appears to be the more common (Dauer and Przedborski, 2003). Currently, the most effective therapy for alleviating the symptoms of PD is levodopa (L-DOPA) (Fahn and Przedborski, 2000), which increases the levels of dopamine in the brain. Although, there is no evidence that levodopa alters the progression of the disease, on one hand, speculations exist that levodopa may actually contribute to the progression of PD (Fahn, 1997; Weiner, 2000) while on the other, it is thought that levodopa is actually neuroprotective (Rajput, 2001) and non-toxic to the human substantia nigra (Rajput, 2001). For reasons that are not yet understood, dopaminergic neurons in the SNpc appear to be more susceptible to damage than other neurons in the brain. Theories as to why this situation exists include genetics (Vila and Przedborski, 2004), excititoxicity (Olanow and Tatton, 1999), inflammation in the brain due to changes in the neuronal environment (Langston et al, 1999; Hunot and Hirsch, 2003, Teismann et al, 2003), protein aggregation (Ii et al, 1997; Trojanowski et al, 1998) oxidative stress, (Fahn and Cohen, 1992;

Przedborski and Jackson-Lewis, 2000), and growth factor (neurotrophin) decline (Mogi et al, 1999; Nagatsu et al, 2000).

About 10% of the PD cases are familial. To date, a number of genetic mutations have been found both in multiple pedigrees and in single families. Multiple pedigree mutations include those found in the alpha-synuclein, parkin, Nurr-1 (nuclear receptor related-1) and DJ-1 genes whereas UCHL-1 (ubiquitin c-terminal hydrolase-1) and NF-M (neurofilament medium) gene mutations have been localized to single families (Huang et al, 2004). Most, if not all of these identified genes function for the survival of the dopamine neuron (synthesis, metabolism, energy supply, cellular detoxification). Thus, any mutation in these genes could lead to misfunctions in the dopamine neuron making them more susceptible to such problems as energy crisis and oxidative stress that could lead to eventual death. In addition to these PD-inducing mutations, several mechanisms have been proposed regarding the etiology of PD. These include ion homeostasis, neuroinflammation, protein aggregation and alterations in growth factors.

Calcium homeostasis is important to normal dopamine neuron function. The NMDA (N-methyl-D-aspartate) and the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors, both ionotrophic glutamate receptors, are, in part, responsible for intracellular calcium homeostasis (Rego and Oliveira, 2003). Overstimulation of these glutamate receptors can alter local calcium homeostasis. Calcium is known to up-regulate enzymes like phospholipase A_2, nitric oxide synthase and xanthine oxidase, all of which are found in mitochondria

and all can stimulate reactive oxygen species (ROS) production. Thus, if local calcium control is compromised resulting in an excitatory-stimulated release of ROS and if existing antioxidant systems cannot handle the produced ROS, mitochondrial dysfunction and damage to several synaptic and intracellular proteins ensues.

Progression of a number of neurological diseases has been shown to be related to inflammation in the brain which can affect the neuronal environment. For instance, multiple sclerosis is a neuroinflammatory disease that causes a loss of the myelinated tracts in the CNS (Hafler, 2004) and recent evidence has shown that there is an inflammatory component to amyotrophic lateral sclerosis (Drachman et al, 2002). Furthermore, supporting a role for inflammation in PD is the finding by Langston and colleagues that brains from individuals, who died from a PD-like syndrome resulting from the self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and who lived for 3-16 years following exposure, showed a significant presence of activated microglia (Langston et al, 1999), thus the theory of inflammation in the SNpc. However, inflammation, thus far, has not been shown to be the cause of PD but rather it is suggested that inflammation may be instrumental in its progression.

Although there is still much debate on the subject, the finding of protein aggregates (both intracellular and extracellular) in many of the neurodegenerative diseases including PD has led to the hypothesis that the improper disposition of proteins may be toxic to the dopamine neuron and can contribute to the neurodegenerative process. In the simpliest of terms, the ubiquitin-proteasome

system (UPS) is like a sink which degrades both abnormal and damaged proteins in the neuron. Proteins are first ubiquinated by the covalent attachment of a polyubiquitin chain and then the whole complex is degraded by the 26S proteasome (Vigouroux et al, 2004). If this system fails to operate properly, it is thought that the aggregation of proteins to be disposed of follows. For example, Lewy bodies are a pathological hallmark of PD and they contain significant amounts of modified alpha-synuclein (Dauer and Przedborski, 2003). Recent reports have shown that aggregated alpha-synuclein not only binds to but also inhibits ubiquitin-dependent proteasomal function (Snyder et al, 2003). Furthermore, oxidized proteins can accumulate in the neuron and this abnormal accumulation of proteins may be toxic enough to put the neuron in an oxidative stress situation which is a highly damaging event.

The growth factor decline hypothesis begs the question why are these substances decreased in the SNpc of PD brains. Growth factors (neurotrophins) are proteins that are normally highly expressed in the substantia nigra (SN) and several lines of evidence demonstrate a decrease in growth factors, particularly glial-derived neurotrophic factor (GDNF) and brain-derived growth factor (BDNF) in the SN of PD brains (Chauhan et al, 2001). The reason for these decreases remain unknown. And, there is nothing known about growth factor decline and oxidative stress. What is clear is that the most of the afore-mentioned hypotheses involve some kind of oxidative stress situation. We and others have used MPTP to follow the oxidative stress hypothesis and the proposed roles of superoxide, nitric oxide and dopamine in the vulnerability of the dopamine neuron (Figure 1).

The MPTP Neurotoxic Process

As a highly lipophilic compound, MPTP can be absorbed through the skin, be injested, injected and snorted. However administered, MPTP rapidly crosses the blood brain barrier and is taken up into glial cells by monoamine (Brooks et al, 1989) and glutamate transporters (Hazell et al, 1997) or pH-dependent antiporters (Marini et al, 1992; Kopin et al, 1992). Once in glial cells, MPTP is converted to MPDP+ (1-methyl-4-phenyl-2,3-dihydropyridinium) by monoamine oxidase-B (MAO-B) and then to MPP+ (1-methyl-4-pyridinium) (Ransom et al, 1987) by spontaneous oxidation. Since MPP+ is a polar compound, it cannot cross membranes; it is speculated that MPP+ is extruded from glia via some kind of transport system. Evidence for a role of glia in the conversion of MPTP to MPP+ comes from Brooks et al (1989) who demonstrated that fluoxetine, a serotonin uptake inhibitor, attenuates MPTP-induced dopaminergic toxicity but does not interfere with MPTP metabolism. Following extrusion into the extracellular space, MPP+ is taken up into the dopamine neuron by the dopamine transporter (DAT) (Kostic et al, 1996). This transporter may be damaged in the MPP+ uptake process as recent evidence by Jakowec at el (2004) have shown that the number of DAT in the substantia nigra following MPTP administration is decreased. MPTP targets primarily DA neurons and the syndrome it produces, over a period of about a week (Jackson-Lewis et al, 1995), is reminiscent of end-stage PD. MPTP causes a far greater loss of DA neurons in the SNpc than of those DA neurons in the ventral tegmental area. It also produces about 90% degeneration of DA nerve terminals in the striatum (Jackson-Lewis, et al, 1995). In order for this level of

damage to occur in the nigrostriatal dopaminergic pathway, MPP+ has to stimulate or recruit compounds from within this pathway.

MPTP and Glial Cells

The non-neuronal support system in the CNS are the glial cells (Abbott, 1988). Under physiological conditions, glia secrete substances into the extracellular environment that support the normal functioning of the neuron (Abbott, 1988). For instance, not only is it known that microglia remove debris from the neuronal environment but, depending on the situation, they can be a source of neurotrophic and neuroprotective molecules such as interleukin-6, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor and epidermal growth factor. On the other hand, they can also produce neurotoxic compounds like nitric oxide, superoxide, tumor necrosis factor, glutamate, arachidonic acid and proteolytic enzymes (Banati et al, 1993). Astrocytes seem to exert a protective effect on dopaminergic neurons as it has been demonstrated that they can produce neurotrophins like nerve growth factor, ciliary growth factor and interleukin-6 (Muller et al, 1995) as well as GDNF (Bohn, 1999). What is extremely interesting about these glial cells is that they may represent a double-edged sword when it comes to MPTP for in the MPTP neurotoxic process, it is in glia that MPTP is metabolized to MPP+ by MAO-B and microglia produce molecules such as the superoxide radical and nitric oxide which are toxic to dopamine neurons.

MPTP, Superoxide and the SNpc Environment.

The environment surrounding SNpc neurons can control the fate of these cells. For example, following MPTP administration, both the extracellular and the intracellular environments of the SNpc DA neuron are altered in such a way that they are no longer part of a supportive system but rather contain detrimental components. Our early studies using transgenic mice that overexpress the copper-zinc form of superoxide dismutase (CuZnSOD) and that were treated with MPTP show that the SNpc of these mice was protected against the damaging effects of MPTP (Przedborski et al, 1992), thus implying the involvement of the superoxide radical. Furthermore, Wu et al (2003) have shown that the infusion of SOD1 into the striatum of MPTP-treated mice is neuroprotective to SNpc neurons, which defines a role for the superoxide radical in the MPTP neurotoxic process. Since CuZnSOD is an extracellular enzyme (Fridovich, 1995), these results suggest that the extracellular environment of the DA neuron is perturbed or altered by the superoxide radical early in the neurotoxic process.

A significant source of the superoxide radical in the extracellular environment is NADPH oxidase (Wu et al, 2003; Gao et al, 2003). NADPH oxidase is a multimeric microglial enzyme that is composed of a number of subunits that include $gp91^{phox}$, $p22^{phox}$, $p47^{phox,}$ and $p40^{phox}$ (Babior, 1999). In resting microglia, this enzyme is inactive because $gp91^{phox}$ and $p22^{phox}$ are separated from the other phox subunits. However, following MPTP administration to mice, the NADPH oxidase complex within the microglia becomes activated because the p47 phox subunit is phosphorylated followed by the movement of the whole complex to the

microglial membrane where it assembles with $gp91^{phox}$ and $p22^{phox}$. This makes the NADPH oxidase complex able to stimulate the production of the superoxide radical. Wu et al (2003), using hydroethidium injections in MPTP-treated mice, visualized the presence of the superoxide radical within microglia located in the SNpc environment of these mice. Up-regulation of NADPH oxidase in postmortem SNpc tissues from PD brains was also shown (Wu et al, 2003). The superoxide radical is then extruded into the extracellular environment where its presence not only alters the neuronal environment but also stimulates the production of secondary oxidants (Babior, 1999) which can, in turn, influence the integrity of the DA neuronal membrane, enter the DA neuron and affect its internal environment.

DA neurons, as abundant as they are in the SNpc. are likely a victim of their own environment. Once MPP+ exits the glial cells, it is taken up from the extracellular space into the DA neuron via the DA transporter (DAT) (Javitch et al, 1985; Bezard et al, 1999). Although recent evidence shows that these transporters are injured during the uptake process (Jakowec et al, 2004), enough of them remain to transport MPP+ into the cytosol of the DA neuron. DAT are absolutely necessary for the MPTP neurotoxic process as several groups (Gainetdinov et al, 1997; Bezard et al, 1999) have shown that MPTP does not harm mice lacking DAT. In the cytosol of the DA neuron, when MPP+ is not taken up into the vesicles, MPP+ can assist in altering the internal environment of the DA neuron by blocking the METC at the complex I site (Nicklas et al, 1985; Nicklas et al, 1987). The major organelle within the DA neuron that produces the lion's share of superoxide

radicals is the mitochondrion (Beal, 2003). This organelle controls oxidation-reduction reactions and are a major source of cellular energy through its oxidative phosphorylation reactions (Przedborski and Jackson-Lewis, 2000). At the complex I site of the mitochondrial respiratory chain (METC), the superoxide radical is released into the cytosol where, under physiological conditions, it is controlled by the manganese form of SOD (MnSOD), which is located in the internal membrane of the mitochondrion (Keller et al, 1998). Many investigators have found a decrease in complex I in various tissues including brain tissue from PD patients (Mizuno et al, 1989; Shapira, 1990). Thus, low activity of complex I in the METC translates to increased production of superoxide radicals, a depletion of MnSOD and an oxidative stress within the DA neuron. An overabundance of superoxide radicals, as stimulated by the presence of MPP+, apparently can no longer be controlled by MnSOD. Klivenyi and colleagues (Klivenyi et al, 1998) have shown that, as long as sufficient stores of MnSOD are present: 1) mice are protected against the damaging effects of MPTP; and 2) the superoxide radical influences the internal environment of the DA neuron. Furthermore, MPP+ has also been shown to affect complex III (Mizuno et al, 1988) such that the increased production of the superoxide radical here also contributes to the disruption of the normal cytosolic environment within the SNpc DA neuron. The relevance of this particular scenario to PD is not well understood because it is not clear whether the deficit in complex I is or is not a cause of PD.

MPTP, Nitric oxide and the SNpc Environment

Nitrative stress related to NO has been documented in PD brains through demonstration of the presence of the inducible form of nitric oxide synthase (iNOS) (Hunot et al, 1996; Hunot et al, 1999) and has been tied, in part, to the activated glia in the vicinity of SNpc DA neurons. Evidence of the up-regulation of iNOS in glia following MPTP administration points to an indirect process rather than a direct up-regulation of this enzyme (Ciesielska et al, 2003). In glia within in the area of the SNpc and the striatum, MPP+ stimulates the up-regulation of proinflammatory cytokines such as TNF-α, interleukin-1-Beta (IL-1β) and interleukin-6 (IL-6) (Youdim et al 2002; Teismann et al, 2003) in a time-dependent manner (Hebert et al, 2003) as early as 12-18 hours prior to the induction of iNOS (Hunot et al, 1999). Immunohistochemical studies (Liberatore et al, 1999; Dehmer et al, 2000) show that iNOS up-regulation occurs in microglia 24 hours after the administration of MPTP which suggests that the proinflammatory cytokines may stimulate the up-regulation of the iNOS enzyme and thereby increase the production of NO within the glia. In a personal communication, Wu and Przedborski (Personal Communication) noted that endothelial NOS (eNOS) is found in the brain vasculature and does not contribute to the MPTP neurotoxic process. Furthermore, neuronal NOS (nNOS), shown to be decreased within non-DA neurons in the SNpc following MPTP administration (Watanabe et al, 2004), probably contributes to the alterations in the intraneuronal rather than the extraneuronal SNpc space because of its location. Moreover, since nNOS knockout mice were only partially protected against the damaging effects

of MPTP whereas 7-nitroindazole offered greater protection (Przedborski et al, 1996), it is likely that nNOS is also a contributor to NO presence in the extracellular space and to the alterations in the extraneuronal environment of the DA neurons in the SNpc.

NO is not a free radical, is highly lipophilic, can readily traverse membranes without the need of a transport system and has the ability to travel as far as 300 microns from its site of production (Lancaster, 1996). Under physiological conditions, both nNOS and iNOS produce significant amounts of NO that are ever present in the extracellular space while levels of the superoxide radical, constantly produced in many biological reactions within the brain, are kept in check by the abundance of SOD. In the pathology of PD and in the MPTP model, increased amounts of the superoxide radical and NO are pushed into the extracellular milieu surrounding the DA neuron. Here, they can react with each other at a faster rate than the superoxide radical can be dismutated by the extracellular CuZnSOD to produce the most damaging secondary oxidant peroxynitrite (Przedborski et al, 2000). Peroxynitrite can damage neuronal membrane proteins and lipids (Przedborski et al, 2000). Thus, the extracellular neuronal environment of SNpc is disturbed or compromised and is no longer supportive for the DA neuron either in PD or in the MPTP model.

Although the superoxide radical does disturb the internal environment of the DA neuron, it is, by itself, not overwhelmingly toxic. In the internal milieu of the SNpc DA neuron, aside from affecting the METC, MPP+ has been demonstrated to increase the expression of the cyclooxygenase-2 (COX-2) enxyme (Teismann

et al, 2003). COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to PGH_2 which is then further metabolized to PGE_2 (O'Bannion, 1999). The NO present in the SNpc DA neuron following MPTP administration most likely enters the DA neuron after having traveled some distance from its non-DA neurons in the SNpc that contain nNOS. When both the superoxide radical and NO are in excess in the internal milieu of the DA neuron after MPTP exposure, PGE_2 catalyzes the reaction between these two relatively mildly toxic compounds to produce the secondary oxidant peroxynitrite (Ischiropoulos and al-Mehdi, 1995; Przedborski and Vila, 2003) which again creates a severely hostile environment for the DA neuron. Peroxynitrite nitrates internal cellular components such as enzymes, fatty acids, proteins, lipids, amino acids and DNA (Radi et al, 2002) of which one of the most important of these is the tyrosine hydroxylase (TH) enzyme. This enzyme is the rate-limiting enzyme in the synthesis of DA and is either down-regulated or damaged in PD and in the MPTP model such that the production of DA is severely compromised (Ara et al, 1997).

DA Toxicity and the SNpc Environment.

The DA neuron in the SNpc may indeed be, at least in part, a contributor to its own death. Following MPTP administration, huge amounts of DA are released from intracellular stores into the extracellular space (Lau et al, 1991; Schmidt et al, 1999). Once released, DA is either enzymatically metabolized by monamine oxidase-B to 3,4-dihydroxyphenylacetic acid and in the process, the hydroxyl radical is kicked out (Burke et al, 2004) or it auto-oxidizes to form a number of

toxic compounds including 6-hydroxydopamine (Graham, 1978) .6-hydroxydopamine is a known neurotoxin that has been used extensively for animal models in PD research (Jeon et al, 1995; Przedborski et al, 1995). It has been demonstrated that this compound destroys striatal DA terminals which results in the death of SNpc DA neurons (Przedborski et al, 1995). Interestingly, one of the findings in PD and in the MPTP model is that there is a greater loss of striatal DA nerve terminals than DA cell bodies in the SNpc (Fahn and Przedborski, 2000). This may be related to the huge release of DA from the storage vescicles caused by the uptake of MPP+. Furthermore, although 6-hydroxydopamine has never been found in brain tissues from PD patients nor in brains from the MPTP model, one can speculate on the possibility that 6-hydroxydopamine or a similarly related compound may contribute negatively to the external environment that surrounds the DA neuron since DA is susceptible to hydroxyl radical (secondary oxidant) attack (Cohen, 1984). A more interesting scenario, however, has been proposed with 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is the intermediate DA metabolite that has been shown to be neurotoxic (Burke et al, 2003). To demonstrate that it is DOPAL and not DA that is neurotoxic, Burke and colleagues (Burke et al, 2003) injected varying concentrations of both compounds into the SNpc of rats. These researchers showed that DOPAL was 5-10 times more neurotoxic than DA. Thus, in the extracellular space, because MAO-B metabolizes DA to DOPAL (Fornai et al, 2000; Burke et al, 2004), DA via DOPAL, possibly contributes to changes in the

extracellular milieu. DOPAL may also be the reason why DA terminals are severely damaged.

In the internal metabolism of the DA neuron, DA can be oxidized to DA-o-quinone and further to 5-cysteinyldopamine (Hastings, 1995). Aside from having a role in peroxynitrite formation through its stimulation of PGE_2, the COX-2 enzyme can facilitate the oxidation of DA which can damage protein-bound sulfhydryl groups (Hastings, 1995). Using HPLC analysis, Teismann et al (2003) showed that MPTP administration elevates ventral midbrain 5-cysteinyldopamine, which is considered a stable modification of DA and evidence that the formation of DA-o-quinone has occurred. DA-o-quinone can contribute to the upheaval of the internal neuronal environment through glutathione depletion and the inactivation of TH (Kuhn et al,1999). On the other hand, while DA is metabolized to DOPAL extraneuronally by MAO-B, within the neuron, DOPAL is formed by MAO-A (Burke et al, 2004). Furthermore, DOPAL is the major metabolite of DA in the human brain (Burke et al, 1999) and levodopa, the drug of choice in the treatment of PD, has been shown to elevate significantly levels of DOPAL in the brain (Fornai et al, 2000). As stated earlier, DOPAL has been shown to destroy the DA neuron at concentrations much lower than DA itself (Burke et al, 2003). Whether MPTP can elevate DOPAL levels in the brain and mimic the death of DA nerve terminals as seen in PD remains to be determined.

Conclusions

Environment plays a significant role in the well-being of the DA neuron. Several cell types including glia and the compounds that these cells secrete work together to maintain an environment suitable for DA neuron survival. Yet, at the same time, these same cells and agents, when perturbed such as following MPTP administration, can contribute to the death of the DA neuron through reactions which alter their physiological concentrations in the SNpc thus putting the DA neuron in a compromised (oxidative stress) situation. Interestingly, the major players in both environments are relatively the same as is their interplay. Thus, DA, superoxide and NO may all conspire to keep the DA neuron in a highly sensitive state, and when presented with a catalyst like MPTP, this sensitivity can shift to vulnerability.

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The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model

A Tool to Explore the Pathogenesis of Parkinson's Disease

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ABSTRACT: **Experimental models of dopaminergic neurodegeneration play a critical role in our quest to elucidate the cause of Parkinson's disease (PD). Despite the recent development of "genetic models" that have followed upon the discovery of mutations causing rare forms of familial PD, toxic models remain at the forefront when it comes to exploring the pathogenesis of sporadic PD. Among these, the model produced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has a competitive advantage over all other toxic models because once this neurotoxin causes intoxication, it induces in humans a syndrome virtually identical to PD. For the past two decades, the complex pharmacology of MPTP and the key steps in the MPTP neurotoxic process have been identified. These molecular events can be classified into three groups: First, those implicated in the initiation of toxicity, which include energy failure due to ATP depletion and oxidative stress mediated by superoxide and nitric oxide; second, those recruited subsequently in response to the initial neuronal perturbations, which include elements of the molecular pathways of apoptosis such as Bax; and, third, those amplifying the neurodegenerative insult, which include various proinflammatory factors such as prostaglandins. Herein, these different contributing factors are reviewed, as is the sequence in which it is believed these factors are acting within the cascade of events responsible for the death of dopaminergic neurons in the MPTP model and in PD. How to target these factors to devise effective neuroprotective therapies for PD is also discussed.**

KEYWORDS: **apoptosis; cell death; nitric oxide; neurotoxicity; neurodegeneration; MPTP; Parkinson's disease (PD); reactive oxygen species; superoxide dismutase**

INTRODUCTION

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a byproduct of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from Par-

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kinson's disease (PD).[1] Recognition of MPTP as a neurotoxin occurred early in 1982, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs that, unknown to anyone, were contaminated with MPTP.[2] In humans and nonhuman primates, depending on the regimen used, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD; in nonhuman primates, however, a resting tremor characteristic of PD has been demonstrated convincingly only in the African green monkey.[3] It is believed that in PD the neurodegenerative process occurs over several years, while the most active phase of neurodegeneration is completed within a few days following MPTP administration.[4,5] However, recent data suggest that, following the main phase of neuronal death, MPTP-induced neurodegeneration may continue to progress "silently" over several decades, at least in humans intoxicated with MPTP.[6,7] Except for four cases,[7,8] no human pathological material has been available for study; thus, the comparison between PD and the MPTP model is limited largely to nonhuman primates.[9] Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD,[10] yet there is a resemblance that goes beyond the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area[11,12] and, in monkeys treated with low doses of MPTP (but not in humans), a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus.[13,14] However, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions called Lewy bodies, so characteristic of PD, have not, thus far, been convincingly observed in MPTP-induced parkinsonism;[9] however, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described.[15] Despite these imperfections, MPTP continues to be regarded as an excellent animal model of sporadic PD, and the belief is that studying MPTP toxic mechanisms will shed light on meaningful pathogenic mechanisms implicated in PD.

Over the years, MPTP has been used in a large variety of animal species, ranging from worms to mammals. To date, the most frequently used animals for MPTP studies have been monkeys, rats, and mice.[16] The administration of MPTP through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and neuropathological features. Herein, we will restrict our discussion to mice, since they have emerged as the preferred animals to explore cellular and molecular alterations produced by MPTP, in part because lines of engineered animals that are so critical to these types of investigations are available only in mice.[17]

MPTP MODE OF ACTION

As illustrated in FIGURE 1, the metabolism of MPTP is a complex, multistep process.[18] After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the protoxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine

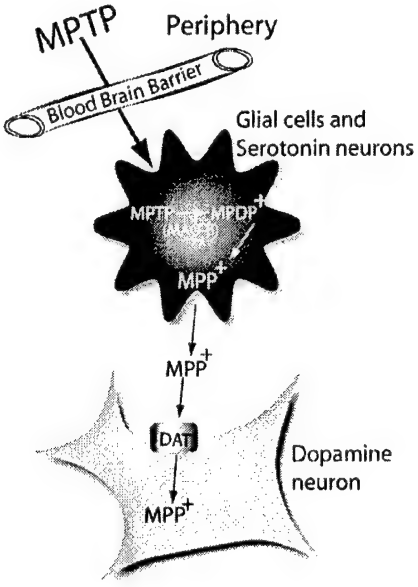

FIGURE 1. Schematic diagram of MPTP metabolism. After its systemic administration, MPTP crosses the blood–brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by monoamine oxidase B within nondopaminergic cells, and then to MPP^+ by an unknown mechanism. Thereafter, MPP^+ is released, again by an unknown mechanism, in the extracellular space. From there, MPP^+ is taken up by the dopamine transporter and thus enters dopaminergic neurons.

oxidase B within nondopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) into the extracellular space. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to dopaminergic neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT),[19] as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol[20] or ablation of the DAT gene in mutant mice[21] completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP.[22]

Once inside dopaminergic neurons, MPP^+ can follow at least three routes (FIG. 2): (1) it can bind to the vesicular monoamine transporters (VMAT), which will translocate MPP^+ into synaptosomal vesicles;[23] (2) it can be concentrated within the mitochondria;[24] and (3) it can remain in the cytosol and interact with different cytosolic enzymes.[25] The fraction of MPP^+ destined to each of these routes is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of

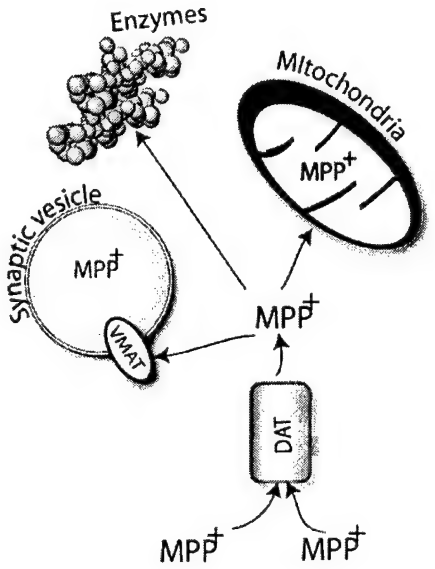

FIGURE 2. Schematic diagram of MPP^+ intracellular pathways. Inside dopaminergic neurons, MPP^+ can bind to the vesicular monoamine transporters, be translocated into synaptosomal vesicles, be concentrated by an active process within the mitochondria, and remain in the cytosol and interact with different cytosolic enzymes.

MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells.[23] Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates.[26] These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (that is, VMAT density) and the magnitude of MPTP neurotoxicity. Inside dopaminergic neurons, MPP^+ can also be concentrated within the mitochondria (FIG. 2),[24] where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain[27,28] through its binding at or near the site of the mitochondrial poison rotenone.[29,30]

MPTP MECHANISM OF ACTION

Currently, it is believed that the neurotoxic process of MPTP is made up of a cascade of deleterious events, which can be divided into early and late neuronal perturbations and secondary nonneuronal alterations. All of these, to a variable degree and at different stages of the degenerative process, participate in the ultimate demise of dopaminergic neurons.

Early Events

Soon after its entry into dopaminergic neurons, MPP^+ binds to complex I and, by interrupting the flow of electrons, leads to an acute deficit in ATP formation. It appears, however, that complex I activity must be reduced >70% to cause severe ATP depletion in nonsynaptic mitochondria[31] and that, in contrast to *in vitro* MPTP, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels,[32] raising the question as to whether an MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS), especially of superoxide.[33–35] A recent demonstration[36] showed that early ROS production can also occur in this model from the autooxidation of dopamine resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol. The importance of MPP^+-related ROS production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1), a key ROS-scavenging enzyme, are significantly more resistant to MPTP-induced dopaminergic toxicity than their nontransgenic littermates.[37] However, several lines of evidence support the concept that ROS exert many or most of their toxic effects in the MPTP model in conjunction with other reactive species such as nitric oxide (NO)[38–41] produced in the brain by both the neuronal and the inducible isoforms of the enzyme NO synthase.[42,43] Comprehensive reviews of the source and the role of NO in the MPTP model can be found in Przedborski and Vila[1] and in Przedborski and Dawson.[44]

Late Events

In response to the variety of functional perturbations caused by the depletion in ATP and the production of ROS, death signals, which can activate the molecular pathways of apoptosis, arise within intoxicated dopaminergic neurons. Although at this time we cannot exclude with certainty the possibility that apoptotic factors are in fact always recruited regardless of MPTP regimen, only prolonged administration of low-to-moderate doses of MPTP is associated with definite morphologically defined apoptotic neurons.[5,45] Supporting the implication of apoptotic molecular factors in the demise of dopaminergic neurons after MPTP administration is the demonstration that the proapoptotic protein Bax is instrumental in this toxic model.[46] Overexpression of the antiapoptotic Bcl-2 also protects dopaminergic cells against MPTP-induced neurodegeneration.[47,48] Similarly, adenovirus-mediated transgenic expression of the X chromosome–linked inhibitor of apoptosis protein (XIAP), an inhibitor of executioner caspases such as caspase-3, also blocks the death of dopaminergic neurons in the SNpc following the administration of MPTP.[49,50] Additional caspases are also activated in MPTP-intoxicated mice such as caspase-8, which is a proximal effector of the tumor necrosis factor receptor (TNFr) family death pathway.[51] Interestingly, however, in the MPTP mouse model it is possible that caspase-8 activation is consequent to the recruitment of the mitochondria-dependent apoptotic pathway and not, as in many other pathological settings, to the ligation of TNFr.[52] Other observations supporting a role of apoptosis in the MPTP neurotoxic process include the demonstration of the resistance to MPTP of the fol-

lowing: mutant mice deficient in p53,[53] a cell cycle control gene involved in programmed cell death; mice with pharmacological or genetic inhibition of c-Jun N terminal kinases;[54–56] or mice that received a striatal adenoassociated virus vector delivery of an Apaf-1–dominant negative inhibitor.[57] Collectively, these data show that during the degenerative process the apoptotic pathways are activated and contribute to the actual death of intoxicated neurons in the MPTP model.

Secondary Events

The loss of dopaminergic neurons in the MPTP mouse model is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes.[58] From a neuropathological standpoint, microglial activation is indicative of an active, ongoing process of cell death. The presence of activated microglia in postmortem samples from MPTP-intoxicated individuals who came to autopsy several decades after being exposed to the toxin[59] suggests an ongoing degenerative process and thus challenges the notion that MPTP produces a "hit and run" kind of damage. Therefore, this important observation[59] suggests that a single acute insult to the SNpc by MPTP could set in motion a self-sustained cascade of events with long-lasting deleterious effects. With mice injected with MPTP and killed at different time points thereafter, it appears that the time course of reactive astrocyte formation parallels that of dopaminergic structure destruction in both the striatum and the SNpc, and that glial fibrillary acidic protein (GFAP) expression remains upregulated even after the main wave of neuronal death has passed.[60–62] These findings suggest that, in the MPTP mouse model,[63] the astrocyte activation is secondary to the death of neurons and not the reverse. This conclusion is supported by the demonstration that blockade of MPP^+ uptake into dopaminergic neurons completely prevents not only SNpc dopaminergic neuronal death but also GFAP upregulation.[64] Remarkably, activation of microglia, which is also quite strong in the MPTP mouse model,[60–62,65] occurs earlier than that of astrocytes and, more important, reaches a maximum before the peak of dopaminergic neurodegeneration.[62] In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc clearly occurs within a time frame allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in PD. Activated microglial cells can produce a variety of noxious compounds, including ROS, reactive nitrogen species (RNS), proinflammatory cytokines, and prostaglandins. Observations showing that blockade of microglial activation mitigates nigrostriatal damage caused by MPTP supports the notion that microglia participate in MPTP-induced neurodegeneration.[66] Among specific deleterious factors, cyclooxygenase type-2 (Cox-2) has emerged as an important determinant of cytotoxicity associated with inflammation.[67,68] In the normal brain, Cox-2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature,[69] which suggests a role for Cox-2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, Cox-2 expression in the brain can increase significantly, as does the level of its products (for example, prostaglandin E_2, or PGE_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, Cox-2 promoter shares many features with inducible nitric oxide synthase (iNOS) promoter;[70] thus, these two enzymes are often coex-

pressed in disease states associated with gliosis. Therefore, it is not surprising to find Cox-2 and iNOS expressed in SNpc glial cells of postmortem PD samples;[71] PGE_2 content is also elevated in SNpc from PD patients.[72] Of relevance to the potential role of prostaglandin in the pathogenesis of PD is the demonstration that the pharmacological inhibition of both Cox-2 and Cox-1[73] and the genetic ablation of Cox-2 attenuates MPTP neurotoxicity.[74]

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Genetic ablation of COX-2 attenuates dopaminergic programmed cell death in experimental parkinsonism.

Parkinson's disease (PD) is a neurodegenerative disorder of uncertain pathogenesis mainly characterized by the loss of nigrostriatal dopaminergic (DA) neurons of the substantia nigra pars compacta (SNpc), which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We have previously shown that targeting of cyclooxygenase type-2 (COX-2) attenuated MPTP-induced DA neurodegeneration by mitigating MPTP-induced oxidative damage (Teismann et al., 2003). Here, we investigated whether COX-2 was also involved in the apoptotic death of SNpc DA neurons induced by a sub-acute regimen of MPTP intoxication (30 mg/kg per day for 5 consecutive days). At the peak of apoptotic DA cell death induced by this regimen of intoxication (day 4 after the last MPTP injection), ablation of COX-2 significantly attenuated the number of SNpc apoptotic cells (40% less of SNpc apoptotic cells). To determine whether the reduction of apoptotic cell death resulted in an increased survival of DA cells in this model, we then determined by stereology the number of SNpc tyrosine-hydroxylase positive cells in COX-2-deficient mice, compared to their wild-type littermates. At day 21 after the last MPTP injection, the number of surviving SNpc DA cells was significantly higher in COX-2-deficient mice (75% of saline-injected animals) than in their wild-type littermates (50% of saline injected animals). Overall, these findings indicate that COX-2 may play a role in the activation of apoptotic molecular pathways that leads to apoptotic DA cell death in the MPTP mouse model of PD.

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